Investigating the molecular underpinnings of major depressive disorder and the utility of biomarkers in the inflammatory cytokine pathway as aids for clinical diagnosis and treatment selection

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Abstract

Major depressive disorder (MDD) is a complex, heterogeneous disorder characterised by a pathological distortion of emotional mood. There is evidence of both genetic and environmental risk factors for MDD, and gene-environment interactions may play a particularly important role. Clinically, MDD is defined by patients meeting a number of diagnostic criteria. However, the heterogeneous nature of the disorder can make MDD difficult to diagnose, especially as it shares close similarities with other psychiatric illnesses such as bipolar disorder. Another clinical problem is that antidepressants, the first line of treatment for MDD, are ineffective in a significant proportion of patients. The projects in this thesis address four aims: (i) the identification of novel gene-environment interactions which may increase risk for MDD; (ii) the identification of diagnostic biomarkers for MDD; (iii) the identification of biomarkers for the prediction of treatment response to antidepressants; and (iv) the identification of transcriptional changes associated with antidepressant treatment and successful therapeutic response.

Utilising a model of early life stress in two inbred mouse strains, we investigated the transcriptional effects of maternal separation. The top stress by strain interaction was found in the telomerase RNA component gene (*Terc*). We also found that a single nucleotide polymorphism in *TERC* (rs10936599), previously identified as a predictor of telomere length, interacted with childhood neglect to predict MDD in a human case-control cohort.

A study investigating differences in the transcription of inflammatory cytokines in the blood of MDD patients, bipolar disorder patients and controls, revealed disorder-specific differences in chemokine (C-C motif) ligand 24 and C-C chemokine receptor type 6 which specifically differentiated MDD patients. Furthermore, we found that transcription of tumour necrosis factor and its targets in the inflammatory cytokine pathway, and DNA methylation in interleukin-11 could be used to predict antidepressant response amongst MDD patients. Moreover, transcription of ATP-binding cassette sub-family F member 1 was found to increase on antidepressant treatment, with the magnitude of change corresponding to clinical response.

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Statement of Work

- Chapter 1: I wrote all parts of this chapter. Section 1.5.1 has been adapted from a review paper currently under review for publication, which was jointly written by myself and Charlotte Martin, a medical student at King's College London.
- Chapter 2: Dr Cathy Fernandes and Dr Rachel Kember performed the maternal separation protocol. Dr Cathy Fernandes and I performed the microarray study. Dr Leonard Schalkwyk and I performed the microarray pre-processing. I performed all remaining analysis and I wrote all parts of this chapter.
- Chapter 3: I carried out all experimental procedures, statistical analyses and writing of this chapter.
- Chapter 4: I carried out all experimental procedures, statistical analyses and writing of this chapter.
- Chapter 5: I led the majority of the experimental procedures performed in this chapter whilst supervising Sophie Hackinger, a placement student at the SGDP centre. Dr Rebecca Smith set up the Sequenom procedure and provided guidance throughout the project. I performed all statistical analyses and writing of this chapter.
- Chapter 6: I carried out all experimental procedures, statistical analyses and writing of this chapter.
- Chapter 7: I wrote all parts of this chapter.

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β-Actin	ACTB
β2-Microglobulin	B2M
5-Hydroxyindoleacetic acid	5HIAA
Adrenocorticotropic Hormone	ACTH
Analysis of Variance	ANOVA
Arginine Vasopressin	AVP
ATP-binding cassette sub-family F member 1	ABCF1
Beck Depression Inventory	BDI
Bipolar Association Case Control Study	BACCS
Body Mass Index	BMI
Brain Derived Neurotrophic Factor	BDNF
C-C chemokine receptor type 6	CCR6
Chemokine (C-C motif) ligand 24	CCL24
Childhood Trauma Questionnaire	CTQ
Chronic Mild Stress	CMS
Cognitive Behavioural Therapy	CBT
Corticotrophin Releasing Hormone	CRH
Corticotropin releasing hormone receptor 1	CRH1
Cycle Threshold	C_t
Depression Case Control Study	DeCC
Diagnostic and Statistical Manual of Mental Disorders IV	DSM-IV
Dioxyribonucleic Acid	DNA
Early Stressful Life Event	eSLE
Electroconvulsive Therapy	ECT
Emotional Neglect	EN
False Discovery Rate	FDR
FK506-Binding Protein	FKBP5
Food and Drug Administration	FDA
Genome-based Therapeutic Drugs for Depression	GENDEP
Genome-Wide Association Study	GWAS
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Hamilton Rating Scale for Depression	HAM-D
Human genomic DNA Control	gDNA
Hypothalamic-Pituitary-Adrenal	HPA
Hypoxanthine Phosphoribosyltransferase	HPRT1
Indoleamine 2,3 dioxygenase	IDO
Interferon-α	IFN- α
Interleukin 1	IL1
Interleukin-10	IL10
Interleukin-11	IL11
Interleukin-1β	IL1B
Interleukin-6	IL6
International Classification of Diseases 10	ICD-10
Interpersonal Psychotherapy	IPT
Linkage Disequilibrium	LD
Major Depressive Disorder	MDD
Messenger RNA	mRNA

Monoamine Oxidase Inhibitor	MAOI
Montgomery–Åsberg Depression Rating Scale	MADRS
National Institute for Health and Care Excellence	NICE
Noradrenaline Transporter	NAT
Physical Neglect	PN
Polymerase Chain Reaction	PCR
Positive PCR Control	PPC
Quantitative Polymerase Chain Reaction	qPCR
Reverse Transcription Control	RTC
Ribonucleic Acid	RNA
Ribosomal Protein L13A	RPL13A
Ribosomal RNA	rRNA
RNA Integrity Number	RIN
Robust Multi-array Average	RMA
Schedules for Clinical Assessment in Neuropsychiatry	SCAN
Selective Serotonin Reuptake Inhibitor	SSRI
Serotonin transporter	5-HTT
Serotonin-transporter-linked polymorphic region	5-HTTLPR
Single Nucleotide Polymorphism	SNP
Stressful Life Event	SLE
Telomerase RNA Component	TERC
Transfer RNA	tRNA
Tumour Necrosis Factor	TNF
World Health Organisation	WHO

Chapter 1

Introduction

Figure 1.0: Major Depression and the brain. Image adapted from http://www.tricitypsychology.com/major-depression-common-after-traumatic-brain-injury/.

1.1 Major Depressive Disorder

The studies described in this thesis examine the causes of major depressive disorder (MDD), and the utility of peripheral cytokine biomarkers as aids for clinical diagnosis and treatment selection. This section summarizes the current research findings relating to the diagnosis, epidemiology, aetiology, and treatment of MDD, as well as the potential utility of cytokine biomarkers.

1.1.1 Diagnosis of Major Depressive Disorder

MDD is a complex heterogeneous disorder which is characterised by a pathological distortion of emotional mood (Jones et al., 2002). MDD is diagnosed when patients meet a number of clinical diagnostic criteria, as detailed in the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) and International Classification of Diseases 10 (ICD-10). According to the DSM-IV, an individual is diagnosed with MDD if they show persistently lowered mood, loss of interest or pleasure for two weeks or more, along with at least four accompanying symptoms. Accompanying symptoms can include: changes in appetite or weight, sleep changes, psychomotor agitation or retardation, loss of energy, feelings of worthlessness or excessive inappropriate guilt, diminished concentration, and recurrent thoughts of death or suicide. Additionally, symptoms must not meet the criteria for a mixed episode, the effects of a substance or general medical condition, or be caused by bereavement. Furthermore, the symptoms must cause clinically significant distress and cause an impairment in social, occupational or other areas of functioning. In addition to categorical diagnosis, clinical scales have also been developed to measure

depressive symptoms and their severity. Examples include the Beck Depression Inventory (BDI; Beck & Steer, 1984), Hamilton Rating Scale for Depression (HAM-D; Hamilton, 1960), and the Montgomery–Åsberg Depression Rating Scale (MADRS; Montgomery & Asberg, 1979).

1.1.2 Epidemiology of MDD

MDD is estimated to affect 350 million people worldwide, and the World Health Organisation (WHO) predicts MDD to become the second leading cause of disability globally by 2020 (Murray & Lopez, 1996; WHO 2012). The prevalence of MDD is twice as high amongst women as men (Nolen-Hoeksema, 1990; Blazer et al., 1994; Kessler et al., 1994); a feature hypothesized to relate to sex differences in hormones, coping strategies, and tendencies to seek medical help (Ernst & Angst, 1992; Nolen-Hoeksema, 2000; Kessler et al., 2003). Sociodemographic factors have been found to influence lifetime prevalence rates of MDD and age of onset, with high-income countries reporting higher lifetime prevalence rates of MDD (14.6%) and a later age of onset (25.7 years), relative to middle-to low-income countries which show a lower lifetime prevalence rate (11%) and earlier age of onset (24 years) (Bromet et al., 2011). Interestingly, studies focusing on high-income countries only find that those with lower socioeconomic status show increased rates of MDD, relative to those with higher levels of education and income (Lorant et al., 2003).

MDD is a recurring, chronic disorder, with an estimated 80% of those diagnosed with MDD experiencing two or more episodes during their lifetime (Mueller et al., 1999; Hollon et al., 2006). Furthermore, duration of major

depressive episodes, severity of episodes, neurobiological factors, socioeconomic status, and comorbities have all been found to moderate the rate of episode recurrence (Keller et al., 1992; Lorant et al., 2003; Spijker et al., 2004; Hollon et al., 2006; Holma et al., 2008).

1.1.3 Comorbidity with MDD

MDD is associated with high rates of comorbity, both with psychiatric and physical disorders. Studies have revealed that over 50% of MDD patients also qualify for the diagnosis of an anxiety disorder (Löwe et al., 2008). MDD patients are also more likely to suffer from substance abuse, eating disorders, post-traumatic stress, impulsive control disorder, obesity, type-2 diabetes, cardiovascular disease, thyroid disease and osteoarthritis (Katon et al., 2007; Farmer et al., 2008; Harvey & Ismail, 2008).

1.1.4 Genetic Causes of MDD

Studies have revealed that MDD tends to aggregate in families, with one meta-analysis reporting a nearly three-fold increase in susceptibility to MDD amongst first-degree relatives of MDD patients (Sullivan et al., 2000). Similarly, high concordance rates for MDD have been revealed amongst twins, suggesting that genetic factors or a closely shared environment may moderate the risk of MDD. To tease out the contribution of shared environment and shared genetics in MDD, twin studies comparing concordance rates between monozygotic twins (sharing 100% of the same genes) and dizygotic twins (sharing 50% of the same genes) have been employed. Such studies assume that if all twins share the same

environmental upbringing, higher concordance rates between monozygotic twins, relative to dizygotic twins, suggest that genetic factors increase the susceptibility to a trait, which is what has been found for MDD (Kendler et al., 1992). Furthermore, sophisticated modeling has been used to calculate heritability estimates. 'Heritability' refers to the proportion of between-individual differences in vulnerability to a trait explained by the additive effect of genes (Keers et al., 2011). Results from twin modelling studies have revealed a broad range of heritability estimates for MDD (between 29-70%), with broad estimations attributed to differences in study design (clinical versus community based studies) and sample selection (recurrent versus non-recurrent, early versus late-onset) (Kendler et al., 1992; Sullivan et al., 2000; McGuffin et al., 2007). All of these twin studies however, confirm that a substantial proportion of risk to MDD in the population relates to genetic background.

Despite twin studies suggesting MDD has a strong genetic component, genome-wide association studies (GWASs) have not been able to identify particular genes associated with the presence of MDD (e.g. Sullivan et al., 2009; Lewis et al., 2010; Muglia et al., 2010). This includes a recent "mega-analysis" comparing the genes of 9240 MDD cases and 9519 controls which did not identify genes robustly associated with MDD (Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium, 2013). Results therefore suggest that a 'missing heritability' exists, whereby the high heritability estimates from twin studies do not translate to the identification of differences at the molecular genetic level. This may be the result of numerous factors, including: the ineffective definition of the phenotype; the presence of many SNPs with very small effect sizes contributing to MDD; the existence of copy number variations;

epigenetic factors; and gene-environment interactions (Zuk et al., 2012).

1.1.5 Environmental Causes of MDD

The most robust environmental factor associated with MDD is stress (Hosang et al., 2012). 'Stress' describes any environmental change, internal or external, that disturbs the maintenance of homeostasis (Pacák & Palkovits, 2001). Stressful life events (SLEs) refer specifically to events that produce a strain on an adult's life, such as the loss of a job, marital separation, or loss of a loved one (Hosang et al., 2012). Severe SLEs are predicted to occur on average every 3 to 4 years in adult life (Brown et al., 1987). Studies have revealed that SLEs such as the end of a relationship or loss of a loved one, double the risk of MDD (Mazure et al., 1998). One study estimates that approximately 20% of individuals who experience a severe SLE will develop MDD (Brown et al., 1987). SLEs also predict the recurrence of depressive episodes in already diagnosed MDD patients (Kessler, 1997). Subsequently, there are clear links between SLEs, MDD pathophysiology and episode precipitation.

Early stressful life events (eSLEs) in the form of childhood abuse (physical, sexual and emotional) and neglect (physical and emotional) have also been linked to an increased likelihood of MDD in adulthood, in conjunction with long-lasting changes in brain structure and functionality, and an exacerbated stress response (Kaufman & Charney, 2001, Meaney 2001, Essex et al, 2002, Fisher et al., 2013). It is thought that eSLEs alter neurobiological circuitry during 'sensitive' periods of development, evoking these enduring physiological and psychological effects (Meaney, 2001).

Interestingly, it has also been found that eSLEs correlate with SLEs in adulthood, and predict future abusive relationships and financial difficulties (Brown et al., 2008; Zielinski et al., 2009). This is in some agreement with the 'kindling hypothesis' which states that stress preceding the onset of MDD symptoms is usually severe (e.g. childhood maltreatment), and sensitizes the individual to more frequent future stresses and depressive episodes (Monroe & Harkness, 2005). Consequently, it has been proposed that the more 'distal' events occurring in childhood may be important in the prediction of SLEs in adulthood and the occurrence of MDD (Keers & Uher, 2012).

1.1.6 Gene-environment interaction causes of MDD

Studies have suggested that gene-environment interactions may play an important role in the pathophysiology of psychiatric disorders (Lesch, 2004; van Os et al., 2008). Gene-environment interactions may be particularly pertinent in MDD, where SLEs are known to increase the susceptibility of some individuals to MDD, but not others. The presence of gene-environment interactions in MDD may also explain our inability to identify genotypes associated with the disorder from GWASs, as it may be the interaction between genes and environment which is more relevant to its pathophysiology (Uher et al., 2008). Furthermore, this could explain the disparity between high heritability estimates from twin studies and the failure to identify genes in MDD GWASs, i.e. the 'missing heritability' phenomenon (see *Section 1.1.4.*). No studies have yet investigated gene-environment interactions on a genome-wide scale, as a very large sample size is predicted to be required to detect interaction effects after correction for

multiple testing. Consequently, novel susceptibility genes for MDD, which may exist as part of a gene-environment interaction, have not yet been identified. However, four candidate genes have previously been found to interact with SLEs to predict MDD, these include: the serotonin-transporter-linked polymorphic region, brain derived neurotrophic factor, corticotrophin-releasing hormone receptor 1, and FK506-binding protein (Keers & Uher, 2011).

1.1.6.1 Serotonin-transporter

Perhaps the most notable, yet highly contentious example of a gene-environment interaction for MDD was based on results from pioneering research carried out by Caspi and colleagues in 2003, identifying an interaction between the serotonin-transporter-linked polymorphic region (*5-HTTLPR*) and SLEs. In Caspi's study they found that increasing numbers of SLEs increased depression severity scores, but was moderated by a functional variant in the serotonin transporter gene, *5-HTTLPR*. It was found that those homozygous for the S-allele (known to produce lower levels of RNA transcript) suffered from significantly higher levels of depression than those homozygous for the higher-expressing L-allele, in response to an increasing number of SLEs (Caspi et al., 2003). This suggests that the serotonin transporter gene may act as a moderator of SLEs and subsequently an individual's susceptibility to MDD. Although, there have been further studies supporting Caspi's findings (e.g. Karg et al., 2011), others have not (e.g. Risch et al., 2009, Fisher et al., 2012, Tomoda et al., 2013) which may relate to differences in study design (e.g. cross-sectional versus longitudinal, and self-reports versus interviewer-assessed).

More recent studies suggest that perhaps the association between the *5-HTTLPR* and stress is more robust when the stress occurs in childhood (Karg et al., 2011). Studies have shown that childhood neglect and maltreatment, particularly sexual abuse interacts with the *5-HTTLPR* to predispose to MDD in adulthood (Uher et al., 2011; Fisher et al., 2013).

1.1.6.2 Brain Derived Neurotrophic Factor

A non-synonymous SNP, rs6265, in the brain derived neurotrophic factor gene (*BDNF*) which results in the substitution of a valine for a methionine amino acid (known as the "Val66Met" polymorphism), has been found to interact with SLEs to predict MDD status (Kim et al., 2007). BDNF is involved in the survival, development and function of neurons (Huang & Reichardt, 2001). Met carriers are known to exhibit reduced secretion of BDNF (Egan et al., 2003), show smaller hippocampal volumes (Bueller et al., 2006), and exhibit increased vulnerability to MDD in response to childhood abuse (Kim et al., 2007; Aguilera et al., 2009). Additionally, there's evidence that Val66Met interacts with 5-HTTLPR and SLEs to predict MDD as part of a gene x gene x environment interaction (Kaufman et al., 2006; Kim et al., 2007).

1.1.6.3 Corticotropin releasing hormone receptor 1

A key receptor in the stress response, corticotropin releasing hormone receptor 1 (CRHR1), has been found to moderate susceptibility to MDD. Haplotypes within the receptor which binds the corticotropin-releasing hormone have been

found to interact with the experience of childhood maltreatment to predict both cortisol levels (Tyrka et al., 2009) and MDD status (Bradley et al., 2008; Polanczyk et al., 2009; Kranzler et al., 2011).

1.1.6.4 FK506-binding protein

FK506-binding protein (FKBP5) is a downstream mediator of glucocorticoid receptor signalling involved in protein folding, trafficking and immunoregulation (Ising et al., 2008). One SNP (rs1360780) has been found to affect the expression of the gene, resulting in increased glucocorticoid receptor resistance (a reduced sensitivity to ligand-binding and mediation of downstream signalling) and greater stress-induced increases in cortisol (Ising et al., 2008). rs1360780 has additionally been found to interact with childhood trauma to predict MDD, with T-carriers being more vulnerable to the effects of early maltreatment (Appel et al., 2011; Zimmerman et al., 2011).

1.1.7 Epigenetic Causes of MDD

Epigenetic processes, namely DNA methylation and histone acetylation, act in addition to the DNA sequence as a second layer of information mediating the regulation of functional gene expression (Bell et al., 2011). Epigenetic modifications are influenced by a combination of factors, including genomic and environmental factors (e.g. stressful life events), and as such act as one molecular mechanism through which gene-environment interactions occur (Meaney et al., 2001). DNA methylation is a stable yet reversible modification to cytosine

nucleotides on the DNA strand, and recent studies have identified possible differences in DNA methylation in the brains and blood of MDD patients relative to controls (Sabunciyan et al., 2012; Zill et al., 2012). Furthermore, antidepressants and mood-stabilizers have been found to evoke epigenetic changes, and research suggests these changes may be important in mediating antidepressant response in rodents (Menke et al., 2012; Nasca et al., 2012).

1.2 Neurobiological Causes of MDD

Despite failures to identify the causes of MDD from the genetic level, studies investigating protein differences, drug effects, and structural brain differences hint towards some of the possible neurobiological events contributing to MDD (Lanfumey, 2008). Further work is still needed to better understand the precise neurobiological changes involved in MDD. Nevertheless, several neurobiological theories dominate the literature surrounding MDD; although they are by no means mutually exclusive, four of these theories are discussed next.

1.2.1 The monoamine theory of MDD

The first theories on the neurobiological causes of MDD stem from studies on the treatment of the disorder, as opposed to its cause. Imipramine and iproniazid were two compounds developed in the 1950s for their antihistaminergic and antitubercular effects respectively (López-Muñoz & Alamo). Coincidentally, both compounds were discovered to improve patient mood and were subsequently approved by the Food and Drug Administration (FDA) for the treatment of

MDD. Whilst imipramine blocked serotonin and noradrenergic reuptake at the synapse, iproniazid acted as a monoamine oxidase inhibitor, reducing catabolism of the monoamines (serotonin and norepinephrine) within the synaptic cleft (Maj et al., 1984), see Figure 1.1. Therefore, this evidence suggested that increasing monoamine neurotransmission could reduce depressive symptoms (Charney, 1998). This has been the basis of antidepressant drug development to-date, with behavioural responses to these compounds in mice also forming the basis of novel antidepressant drug screening (Powell et al., 2012, see Appendix 1 for full review article).

Naturally, the discovery that monoamines may play a role in 'anti-depression' has also led to the hypothesis that a reduction in monoamine neurotransmission may be involved in the pathophysiology of MDD (Charney, 1998; Elhwuegi, 2004). If this were the case, we would expect monoamine inhibitors such as reserpine, to induce depressive symptoms (Brodie et al., 1957). However, studies have revealed mixed results, with some subjects demonstrating increases in depressive symptoms in response to reserpine, whilst other studies found reserpine to have antidepressant effects (Baumeister et al., 2003). Furthermore, there is no conclusive evidence of increased serotonin metabolites such as 5-Hydroxyindoleacetic acid (5HIAA) amongst MDD patients, which we might expect if these patients were showing reduced serotonin neurotransmission as a result of rapid catabolism. However, there is strong evidence of a reduction of 5HIAA in suicide attempters, suggesting it may be involved in the impulsive suicidality component of MDD found in some patients (Asberg & Träskman, 1981; Brown & Linnoila, 1990). Consequently, although there is no dispute that

monoamines play a key role in the treatment of MDD, it remains unclear as to whether they are involved in its cause.

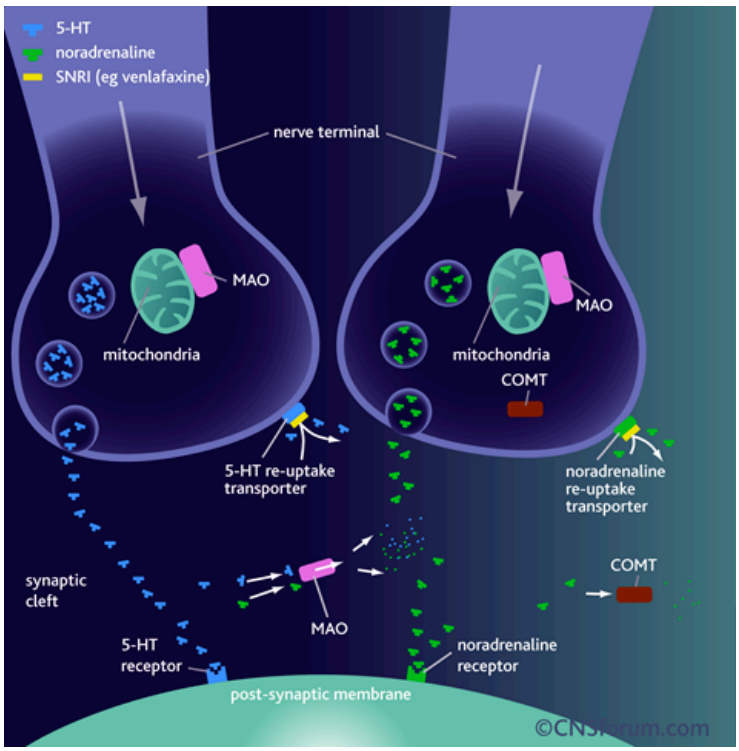


Figure 1.1: Monoaminergic neurotransmission and antidepressant action. The picture shows two pre-synaptic neurons and a post-synaptic neuron. The serotonin transporter (5-HTT), noradrenaline transporter (NAT), and monoamine oxidase (MAO) all represent targets of antidepressant drugs. Antidepressant action results in increased binding of serotonin and noradrenaline at the post-synaptic receptor. Adapted from http://pharmacologycorner.com/differences-between-tricyclic-antidepressants-and-selective-serotonin-norepinephrine-reuptake-inhibitors-mechanism-of-action/.

1.2.2 Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis is the physiological construct governing the stress response. When a mammal is exposed to a stressor, a sequence of events occurs, starting at the paraventricular nucleus of the hypothalamus, where corticotrophin releasing hormone (CRP) and arginine vasopressin (AVP) are released (Lanfumey, 2008). CRP and AVP then bind to receptors on the anterior pituitary, causing the release of adrenocorticotropic hormone (ACTH) which binds to receptors in the adrenal cortex, culminating in the release of the stress hormone cortisol (Lanfumey, 2008), see Figure 1.2. It is estimated that approximately half of patients suffering from MDD show abnormal, excessive activation of the HPA axis, making cortisol levels one of the most robust markers for MDD (Curtis et al., 1976; Hellhammera et al., 2009). Excessive activation of the HPA axis has also been found in humans and mammals exposed to SLEs, particularly eSLEs, suggesting HPA dysregulation may represent a link between stress and MDD (Meaney et al., 2001).

1.2.3 Neurotrophins theory of MDD

The hippocampus is a brain region involved in learning, memory and emotional processing (Strange et al., 1999). Evidence suggests that MDD patients may exhibit volumetric decreases in the hippocampus relative to controls (Sheline et al., 1999; Miller et al., 2000).

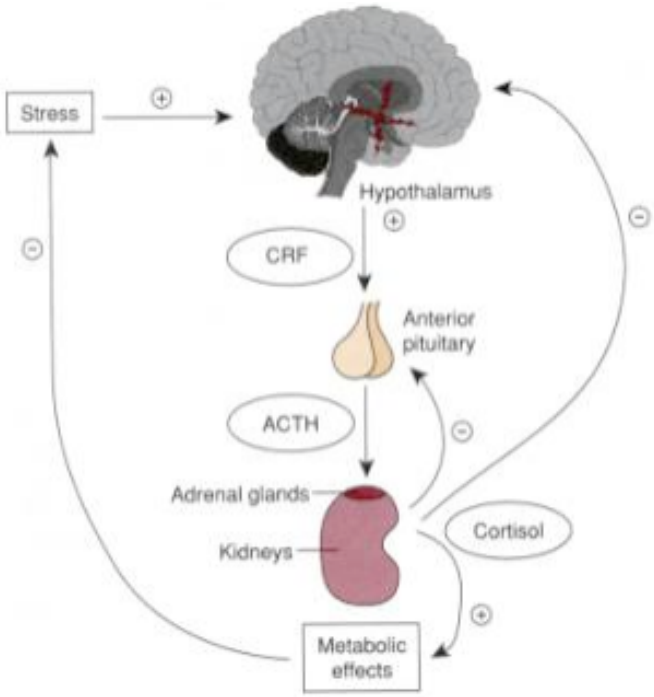


Figure 1.2: The hypothalamic pituitary adrenal (HPA) axis. A summary of the key steps involve in the stress response. Adapted from http://www.montana.edu/wwwai/imsd/alcohol/Vanessa/vwhpa.htm.

Although there are numerous explanations as to the cause of this decreased hippocampal volume (e.g. excessive cortisol release), the most popular theory relates to the neurotrophins (Krishnan & Nestler, 2008). Neurotrophins are a family of proteins involved in the survival, development and function of neurons (Huang & Reichardt, 2001). BDNF has been found to be lower amongst MDD patients, which may contribute to lower hippocampal volumes (Karege et al., 2002). Furthermore, studies have observed increases in BDNF in response to antidepressants (Shimizu et al., 2003), and direct infusion of BDNF into the

brain has been shown to induce antidepressant effects in animal models (Shirayama et al, 2002). It is hypothesized that BDNF is also responsible for promoting the development of neural progenitor cells and hippocampal neurogenesis in response to treatment, with the time taken for this to occur hypothesized to correspond to the 'therapeutic lag' between administration of antidepressants and the alleviation of symptoms (Boldrini et al., 2009).

1.2.4 Cytokine theories of MDD

Cytokines represent a group of cell-signalling molecules which, in the periphery, aid inflammatory processes and the immune system to form co-ordinated responses to infection (Dantzer et al., 2008, see Figure 1.3). However, evidence suggests that they also have potent effects on the brain and may be involved in the pathophysiology of MDD.

1.2.4.1 Cytokines and the brain

Peripheral cytokines can act centrally by accessing the brain through vagal nerve activation or by crossing the blood-brain barrier via 'leaky' regions or as a result of active transport (Banks et al., 1995). Cytokines are also constitutively expressed within the central nervous system and can function both as neural protectors and agents facilitating neurodegeneration (Miller et al., 2009). Cytokines have been found to influence a multitude of systems within the brain, affecting neurotransmitter metabolism, neuroendocrine function and neural plasticity (Miller et al., 2009).

1.2.4.1.1 Cytokines and neurotransmitters

Cytokines have been found to affect both the synthesis of neurotransmitters and neurotransmitter-receptor interactions. For instance, cytokines have been found to activate the enzyme indoleamine 2,3 dioxygenase (IDO), which metabolises tryptophan, the amino acid precursor to serotonin (Fujigaki et al., 2006). Subsequently, the break down of tryptophan culminates in a reduction in the availability of tryptophan for serotonin synthesis (Young & Leyton, 2002). Interestingly, it has also been shown that pharmacological inhibition of IDO prevents the precipitation of sickness behaviours in mice, on treatment with lipopolysaccharide, suggesting the action of cytokines on IDO may be critical for behavioural changes associated with MDD (O'Connor et al., 2008). Cytokines have also been found to affect the functionality of neurotransmitter transporters. For instance, TNF has been shown to enhance serotonin transporter function in human placental cell lines (Mossner et al., 1998), rat neuronal cell lines and in mouse brain (Zhu et al., 2006).

1.2.4.1.2 Cytokines and neuroendocrine function

Acute administration of cytokines has been found to stimulate the HPA axis and subsequently cortisol release (Miller et al., 2009). It is hypothesized that chronic high levels of cytokines may impair the negative feedback loops in the HPA axis resulting in persistently high levels of cortisol release (Besedovsky et al., 1996). This furthermore evokes changes to the glucocorticoid receptor including reduced expression, and a reduced responsiveness to cortisol, as reported amongst MDD patients (Pariante & Miller, 2001).

1.2.4.1.3 Cytokines and neural plasticity

Putative links have also been formed between peripheral immune activation and hippocampal volume (Miller et al., 2009). For instance, peripheral lipopolysaccharide has been associated with heightened intra-hippocampal levels of TNF and IL1, which inhibits expression of BDNF and its receptors, resulting in reduced hippocampal volumes (Wu et al., 2007).

1.2.4.1.4 Cytokines and the pathophysiology of MDD

The first theories linking inflammatory processes to MDD arose as a result of the high rates of comorbidity between MDD and other medical conditions [see *Section 1.1.3*]. A growing body of evidence suggests that cytokines such as the chemokines, interferons, interleukins and the tumor necrosis factor family may have effects of the brain which are important in the pathophysiology of MDD (Dantzer et al., 2008). Patients with MDD who are otherwise healthy have been found to exhibit increased levels of proinflammatory cytokines (e.g. tumour necrosis factor, interleukin-6) and their receptors in peripheral blood, cerebrospinal fluid, and within the brain itself (Lanquillon et al., 2000; Pandey et al., 2011). However, this information alone does not allow us to discern whether abnormal cytokine levels are a cause or effect of MDD, but other lines of evidence do suggest that changes in cytokines may represent the cause of MDD symptoms. For instance, studies have repeatedly confirmed that the pro-inflammatory cytokine interferon-alpha (IFN-a), used in the treatment of hepatitis and cancer, elicits depressive symptoms in a large proportion of patients (~40%), along with altered neurotransmitter metabolism, neuroendocrine function, and

responsiveness to antidepressant pharmacotherapy (Bonaccorso et al., 2002; Raison et al, 2006). These findings linking cytokines to MDD are further supported by animal studies. For instance, rodents injected with immunoreactive substances, such as lipopolysaccharide, display 'sickness behaviours' consisting of behaviours analogous to those exhibited by MDD patient, for example anhedonia, decreased activity, cognitive dysfunction, and altered sleep (Konsman et al., 2002). These observations therefore imply that endogenous cytokines and their modulation of the innate immune system are involved in the pathophysiology of MDD.

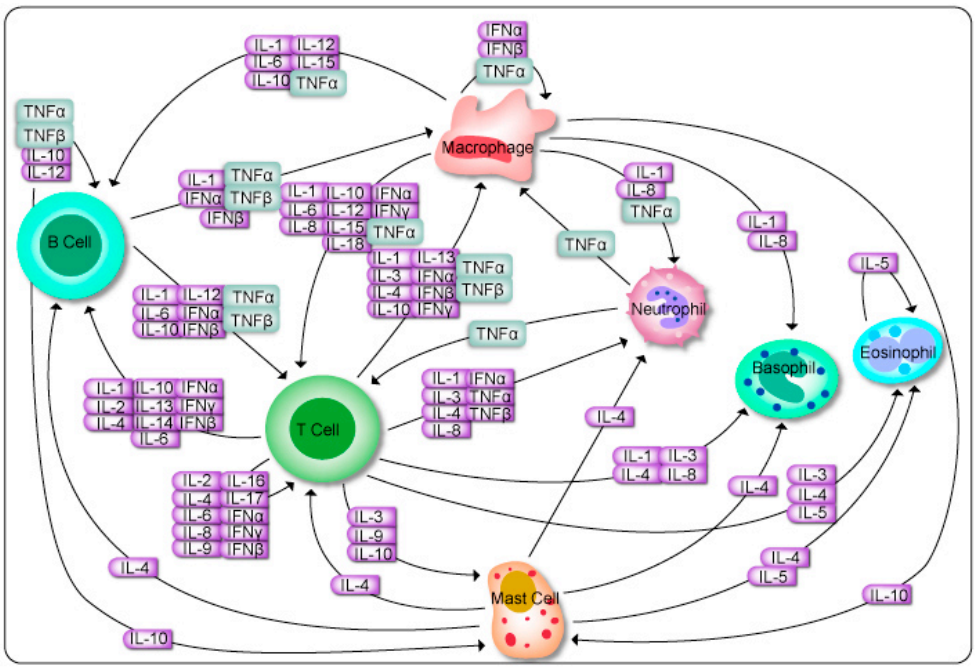


Figure 1.3: The inflammatory cytokine pathway. A picture showing the complex interactions between immune cells, orchestrated through the release of different types of inflammatory cytokines. Adapted from http://www.genecopoeia.com/product/search/pathway/h_cytokinePathway.php.

1.2.5 Mouse models of MDD

Some features of MDD show similarities to mouse behaviours, and, as such, mouse models have been developed to improve our understanding of the pathophysiology and treatment of depressive symptoms. Mouse behaviours such as immobility in the forced swim test and tail suspension tests are thought to mirror behavioural despair present in human depression (Porsolt et al., 2001). Whereas, anhedonia is measured by a decrease in sucrose consumption in the sucrose preference test in mouse (Papp et al., 1991) and by an increase in latency to approach and eat food in the novelty-suppressed feeding test (Dulawa & Hena, 2005); see Powell et al., (2012a) as found in *Appendix A* for more details.

One of the main advantages of using mice in researching a genetic disorder such as MDD is that over 95% of the mouse genome is similar to the human genome (The Jackson Laboratory, 2013). Furthermore, the utilisation of inbred strains and knockouts allow us to test the influence of different genetic backgrounds in response to specific changes to the environment (Powell et al., 2012a). In particular, mouse models can be used to investigate: the effects of stress on behaviour (Meaney et al., 2001), the presence of stress by gene interactions (Kember et al., 2012), the antidepressant properties of novel compounds (Jacobson and Cryan, 2007), and the presence of pharmacogenetic interactions (Malki et al., 2010).

Two common mouse protocols used to model MDD phenotypes in mice include the chronic mild stress, and maternal separation protocol, which are both based on the assumption that stressful events are involved in the cause of MDD (Deussing, 2006). The chronic mild stress (CMS) model of MDD involves exposing animals to numerous mild and unpredictable stressors (Willner, 2005).

Stressors can include temperature changes, changes in amounts of food and water, and changes to their cage mates (Willner et al., 1992). It is hypothesized that the CMS protocol models the daily unpredictable stresses faced in adult human life, which may contribute to MDD (Deussing, 2006). The CMS protocol induces behavioural, neurochemical changes and changes in responses to reward, thought to parallel those changes occurring in MDD patients (Willner, 2005). The maternal separation protocol specifically models early life stress and involves young pups being separated from their dam (mother) for between 1 and 24 hours, either as a single separation or part of a repeated separation over numerous days (Meaney et al., 2001). This protocol induces long-lasting increases in anxiety-like behaviours, increased stress-responsiveness in adulthood, and long-lasting molecular changes in the brain (Meaney et al., 2001; Kember et al., 2012).

1.3 Treatments for MDD

According to the National Institute for Health and Care Excellence (NICE), a combination of pharmacotherapy and high intensity psychological therapy (e.g. cognitive behavioural therapy) is recommended for the treatment of moderate to severe MDD (NICE, 2004). Here we discuss some common pharmacotherapies used to treat MDD, as well as cognitive behavioural therapy, alternative psychological therapies, and electroconvulsive therapy.

1.3.1 Pharmacotherapies used to treat MDD

Antidepressants are the first line of treatment for MDD. As described in *Section*

1.2.1, antidepressants target the monoamines, which are a group of neurotransmitters, derived from aromatic amino acids that have crucial roles in emotion, arousal and cognition (D'Souza & Craig, 2008). Antidepressants are understood to evoke their effects through increasing the neurotransmission of monoamines, particularly serotonin and noradrenaline, across the synapse (Charney, 1998). There are ten classes of antidepressant drugs commonly used to treat MDD, however, two of the most commonly prescribed classes are the selective serotonin reuptake inhibitors and tricyclics (Lawrenson et al., 2000).

1.3.1.1 Selective Serotonin Reuptake inhibitors

Selective serotonin reuptake inhibitors (SSRIs) are compounds which selectively block the serotonin transporter at the pre-synaptic neuron. This results in reduced reuptake of serotonin, allowing more to cross the synapse and bind to postsynaptic receptors (Charney et al., 1998). Although the main use of SSRIs is in the treatment of MDD, they are also used to treat generalised anxiety disorder, obsessive compulsive disorder, eating disorders and premature ejaculation (Stone et al., 2003). The main advantage of SSRIs is their more targeted nature. Unlike the "richer" pharmacology of the older tricyclics which affect numerous receptor and transporter types, SSRIs more selectivity target the serotonin transporter, resulting in lower rates of reported side effects (Lawrenson et al., 2000).

An example of a commonly prescribed SSRI is escitalopram, its structure is shown in Figure 1.4. This drug has the highest affinity amongst all SSRIs for the serotonin transporter, and consists of a single active S-enantiomer. Escitalopram has been claimed to have more potent antidepressant effects than

the closely related racemic mixture citalopram (Montgomery et al., 2001).

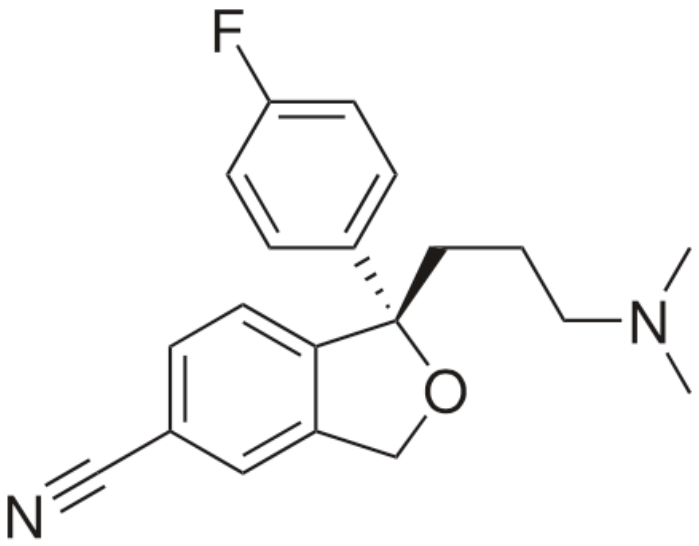


Figure 1.4: The chemical structure of escitalopram.

1.3.1.2 Tricyclics

Tricyclics are named as such due to their three-ringed structure, see Figure 1.5. This class of antidepressant is particularly efficacious in the treatment of depression with melancholic features (Perry, 1996). Due to the richer pharmacology of these drugs (i.e. the fact they target a broad range of receptors and transporters), they are associated with a greater occurrence of side effects, but can also be used in the treatment of a broader range of ailments than other antidepressants such as SSRIs (Lawrenson et al., 2000). As well as their use in MDD and anxiety disorders, tricyclics have also been prescribed for use in chronic pain, Tourette's syndrome, irritable bowel syndrome, narcolepsy and

eating disorders, amongst others (Gillman, 2007).

An example of a commonly prescribed tricyclic antidepressant is nortriptyline, its structure is shown in Figure 1.5. Nortriptyline is a second-generation tricyclic antidepressant with a particularly high affinity for the noradrenaline transporter, with a lesser effect on serotonin and dopamine reuptake (Gillman, 2007).

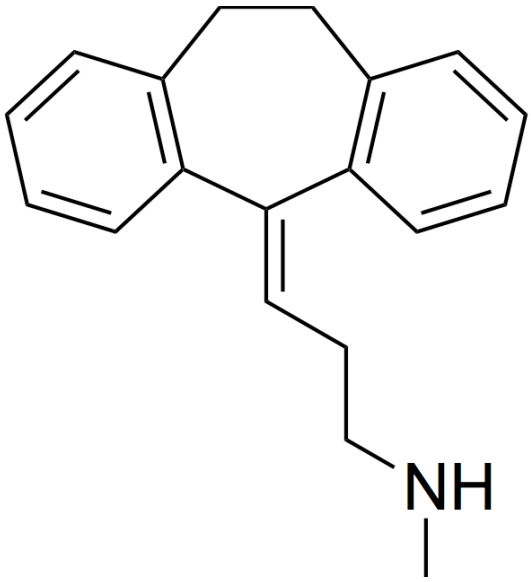


Figure 1.5: The chemical structure of nortriptyline

1.3.2 Psychological Therapies for MDD

Psychological therapies for MDD include cognitive behavioural therapy, interpersonal therapy, psychoanalysis and mindfulness. Cognitive behavioural therapy (CBT) is a form of psychological intervention which employs theory from behavioural and cognitive realms to tackle psychological disorders (Schacter et al, 2010). It works by changing maladaptive thinking, mood, and subsequently

behaviour. Studies have suggested CBT can be as effective as monotherapy antidepressant treatment, with CBT and antidepressant therapy combined being the most effective form of treatment (Keller et al., 2000).

Other psychological therapies include interpersonal psychotherapy (IPT), which is a short course of therapy aimed at dealing with interpersonal issues underlying MDD (Frank, 1971). Alternatively, psychoanalysis is therapy focused on Freud's theories on the importance of early childhood experiences and unconscious internal conflicts (Frank, 1971). Furthermore, of increasing interest as a novel therapy for MDD is 'mindfulness'. Mindfulness is thought to be effective for the treatment of MDD by redirecting patients' attention to the present moment, as opposed to ruminating over past negative thoughts (Segal et al., 2013).

1.3.3 Electroconvulsive Therapy

Electroconvulsive therapy (ECT) involves the precipitation of seizures in patients through the application of an electric shock. NICE recommends ECT only in severe MDD, as it is has been linked to brain damage and memory loss (NICE, 2004). Nevertheless, ECT has been found to be effective in treating MDD, and particularly efficacious in treating MDD in patients who show "treatment-resistance"; i.e. those who fail to respond to numerous forms of pharmacological and psychological therapies. (Pagnin et al., 2004; Mayberg et al., 2005). There is limited understanding as to how ECT works, but the short-lived seizures may help to 'jump start' the brain, disrupting pathological brain circuitry and correcting neurochemical and blood-flow abnormalities (Mayberg et al., 2005).

1.4 Problems with diagnosis and treatment selection for MDD

As discussed in *Section 1.1.1.*, MDD is currently diagnosed based on patients meeting a number of "clinical characteristics" such as reporting lowered mood, loss of interest or pleasure (anhedonia), and sleep disturbances (American Psychiatric Association, 2000). However, due to the heterogeneous nature of MDD, it shares overlapping clinical characteristics with other disorders, which increases the risk of misdiagnosis. Furthermore, even after correct diagnosis there are still issues over which treatment is best for an individual (Uher et al., 2010).

1.4.1 Misdiagnosis between Bipolar Disorder and MDD

Both MDD and bipolar disorder (BPD) are considered "mood disorders", sharing common aetiologies and clinical manifestations (Jones et al., 2002). Similarly to MDD, BPD patients experience episodes of depression (e.g. lowered mood, loss of interest or pleasure, loss of energy), however BPD patients additionally experience episodes of mania (e.g. expanded self-esteem, increased distractibility, talkativeness; American Psychiatric Association, 2000). The diagnostic problem exists when BPD patients enter clinic pre-morbid for manic symptoms, which is commonly the case due to the often later onset of mania, and more frequent occurrence of depressive episodes in BPD (Lewis et al., 2003; Perlis et al., 2005). The pre-manic BPD patient will subsequently meet all the clinical criteria for MDD, be misdiagnosed as suffering from MDD, and be treated with antidepressants. Monotherapy antidepressant treatment given to

BPD patients increases their risk of antidepressant induced mania and cycle-acceleration which can have damaging effects on disease prognosis (Wehr & Goodwin, 1979; Altshuler et al., 1995). Therefore, ways to improve the objectivity and specificity of clinical diagnosis (e.g. establishing disorder-specific biomarkers) would prove valuable in differentiating MDD from other related disorders.

1.4.2 Ineffective treatment

Another major clinical problem relates to the ineffective treatment of MDD. It is estimated that despite antidepressants being given as the first line of treatment for MDD, approximately two thirds of patients fail to respond to the first antidepressant prescribed (Gibson et al., 2010). Furthermore, one third fail to respond to multiple forms of antidepressant therapy (Gibson et al., 2010). This lag between diagnosis and the initiation of effective treatment prolongs the suffering of patients and increases the opportunity for suicide attempts. There are four proposed routes to improving the efficacy of treatment. Firstly, the identification of biomarkers might allow us to predict whether an individual is likely to respond better to one type of antidepressant over another, or whether they may be more suited to alternative forms of therapy such as CBT (Hepgul et al., 2013). Secondly, adjuvant therapies may compliment the actions of antidepressants, increasing their efficacy. For instance, its been found that the tumour necrosis factor antagonist infliximab may be a useful adjuvant for improving response in treatment-resistant MDD patients (Raison et al., 2013). Thirdly, antidepressant therapy combined with psychological therapies may

increase responsiveness, above that obtained with antidepressant treatment alone (Keller et al., 2000). Finally, new therapies targeting different biological systems may simply be more efficacious, and are yet to be discovered (e.g. Catena-Dell'Osso et al., 2012).

1.5. Biomarkers for MDD

A biomarker is defined as a 'characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention' (Atkinson et al., 2001). Biomarkers in psychiatry might offer a more objective way of diagnosing a disorder and selecting for the most appropriate treatment. In the case of a heterogeneous, polygenic disorder like MDD the hunt for biomarkers is particularly complex.

Biomarker information at the genetic, transcriptomic and proteomic biological system levels may provide distinct and clinically useful information relating to MDD. Genotypes are stable, constant features that influence systemic physiological function throughout development and can predispose to certain disease states. Genomic information can therefore be used as state predictors of long-lasting functional differences in the brain (Meyer-Lindenberg et al., 2006), disease manifestation (Janssens & Duijn, 2008) and clinical responses to therapies (Kamali & Wynne, 2010). However, the dynamic and environmentally sensitive nature of cellular gene expression means that transcriptional and proteomic biomarkers may not only function as baseline state predictors, but could also potentially be used in conjunction with changes in behavioural traits as

a prospective measure of MDD prognosis (Riedmaier & Pfaffl, 2013). This is of particular clinical interest if early transcriptional or proteomic changes are found to precede behavioural changes relating to symptom severity or early response to antidepressant drugs.

1.5.1 Inflammatory Cytokines as biomarkers in MDD

As discussed in *Section 1.2.4.,* inflammatory cytokines may play a role in the pathophysiology of MDD. Furthermore, due the fact that cytokines have access to the brain but are peripherally accessible, through migration across the blood-brain barrier, they may possess useful biomarker properties relating to MDD (Banks et al., 1995). Here we will summarise recent literature pertaining to the cytokines at each system level (genetic, transcriptomic, proteomic) and demonstrate how they might be used to inform clinical decisions regarding the diagnosis and treatment of MDD.

1.5.1.1 Cytokine biomarkers for MDD diagnosis

As discussed in *Section 1.1.3*, no single gene has yet been robustly associated with MDD based on results from genome-wide association studies (GWASs) (Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium, 2013). However, candidate gene studies suggest that single nucleotide polymorphism (SNP) differences in inflammatory-related genes may mediate susceptibility to MDD and, as such, future polygenic signatures incorporating this information may have useful diagnostic capabilities. Specifically, MDD case-control studies have revealed differences in genes coding

for interleukin 1B, interleukin 6, interleukin 10, and tumour necrosis factor (Clerici et al., 2009; Haastrup et al., 2012; Altamura et al., 2010; Jun et al., 2003; Luciano et al., 2011; Borkowska et al., 2011).

1.5.1.1.1 Interleukin-1B

IL-1β is a member of the IL-1 cytokine family, encoded by the *IL1B* gene at location 2q14 of chromosome 2 (Yu et al., 2003). IL-1β is an important pro-inflammatory cytokine which has a role in coordination of the immune response and regulation of cell proliferation, differentiation and apoptosis (Baune et al., 2010). *IL1B* variants have been associated with decreased function in the amygdala and anterior cingulate cortex (Baune et al., 2010). These brain regions are involved in memory, emotional processing and reward mechanisms, and impaired functioning in each of these neural pathways have been implicated in the manifestation of MDD symptoms (Hamilton et al., 2012). A genotypic combination of homozygosity for the -31T allele (rs1143627) and -511C (rs16944) within *IL1B* has been associated with recurrent MDD, whereas heterozygosity at both sites is more common in controls (Borkowska et al. 2011). Furthermore, patients diagnosed with MDD who are homozygous for the -511C allele tend to have higher levels of depression severity (Yu et al., 2003). At the transcriptional level, *IL1B* has been reported to be higher in MDD patient blood relative to controls (Tsao et al., 2006; Cattaneo et al., 2012). However, there are mixed results at the protein level, with the latest meta-analysis suggesting IL-1B levels may not be significantly elevated amongst MDD patients (Dowlati et al., 2010).

1.5.1.1.2 Interleukin-6

The human interleukin-6 gene (*IL6*) is organised into 5 exons and 4 introns on the short arm of chromosome 7 (Clerici et al., 2009). IL-6 is a pleiotropic cytokine which plays an important role in the acute phase response and chronic inflammation (Barnes et al., 2011). A SNP, rs1800795, in the promoter region has been associated with differential levels of IL-6 transcription and plasma concentrations. Those homozygous for the G-allele are known to produce higher levels of IL-6 and show higher levels of depression following interferon-a and ribavirin treatment (Bull et al., 2009). Furthermore, studies have consistently shown higher levels of IL-6, both at the transcriptional level (Tsao et al., 2006; Cattaneo et al., 2012), and at the protein level (Lanquillon et al., 2000; Alesci et al., 2005; Raison et al., 2006; Yang et al., 2007; Clerici et al., 2009; Miller et al., 2009; Su et al., 2009; Howren et al., 2009; Dowlati et al., 2010; Hiles et al., 2012; Liu et al., 2012) amongst MDD patients relative to controls. Serum levels of IL6 have also been shown to significantly correlate with the severity of MDD symptoms (Su et al., 2009).

1.5.1.1.3 Interleukin-10

The anti-inflammatory properties of IL-10 are well described, with its main role being that of preventing damage to the host during infection (Pierson & Liston, 2010). IL-10 is produced by B-cells and T-cells, and works to suppress the action of T-cells during inflammation (Pierson & Liston, 2010). The gene encoding IL-10 maps to the 1q31-1q32 region on chromosome 1 and it is highly polymorphic, with the biallelic polymorphisms at positions –1082 (G/A)

(rs1800896), −819 (T/C) (rs1800871), and −592 (A/C) (rs1800872) being associated with the transcription and production of IL-10 (Liu et al., 2010). In light of the established link between inflammation and MDD, it has been hypothesised that MDD patients may be carriers of a 'low-producer' polymorphism of the *IL10* gene. The A/A genotype of the polymorphism at -1082 has subsequently been identified as a 'low-producer' allele, and the distribution of this genotype has indeed been found to be significantly more prevalent in MDD patients than controls (Clerici et al., 2009). Furthermore, baseline peripheral levels of IL-10 (Clerici et al., 2009) have been demonstrated to be lower in MDD patients compared with controls.

1.5.1.1.4 Tumour Necrosis Factor

The *TNF* gene is located within the class III coding region of the major histocompatibility complex on the small arm of chromosome 6 (6p21.1–21.3; Clerici et al., 2009). The A-allele of rs1800629 [-308(G/A)], present in the promoter of the *TNF* gene, has been associated with MDD in a Korean population (Jun et al., 2003). In contrast to this finding, studies in a geriatric Caucasian population linked the G-allele to an increased risk of MDD (Cerri et al., 2009; Cerri et al., 2010). A large candidate gene association study of 1738 MDD patients and 1802 controls polymorphism identified another SNP rs76917 associated with MDD, with those carrying the T-allele being more likely to be cases (Bosker et al., 2011).

In contrast to the somewhat mixed results at the genetic level, studies have fairly consistently reported higher levels of TNF mRNA and protein in the blood

of MDD patients relative to controls (Tsao et al., 2006; Cattaneo et al., 2012; Lanquillon et al., 2000; Tuglu et al., 2003; Raison et al., 2006; Yang et al., 2007; Schmidt et al., 2011; Savitz et al., 2012; Dowlati et al., 2010; Liu et al., 2012). Consequently, it may be that molecular influences at the epigenetic or transcription factor binding level relating to the pathophysiology of MDD make TNF mRNA and protein levels better predictors of MDD status than genotype alone.

1.5.1.2 Cytokine biomarkers for treatment response

Studies have revealed that molecular differences within the inflammatory cytokines may be useful in predicting how well patients will respond to antidepressant treatment. Specifically, the cytokines interleukin-6, tumour necrosis factor, interleukin-1B and interleukin-11 have been found to predict responsiveness to antidepressants.

1.5.1.2.1 Interleukin-6

A major SNP in the *IL6* gene (rs7801617) has been associated with a poor response to the SSRI escitalopram as part of a candidate gene association study (Uher et al., 2010). IL-6 has been shown to have the potential to switch the production of the neurotransmitter serotonin to acetylcholine in raphe neurons (Rudge et al., 1996). This inhibition of serotonin production by IL-6 could offer a possible explanation as to why *IL6* genetic variants affect clinical response to SSRIs, which target the serotonin system. There have been few transcriptomic

studies investigating the links between *IL6* transcription and treatment response, but those that have been performed have failed to show an association (Cattaneo et al., 2012). However, patients who are non-responders to antidepressants reportedly have increased plasma levels of IL6 (Maes et al., 1997; Lanquillon et al., 2000; Raison et al., 2006; Eller et al., 2008).

1.5.1.2.2 Tumour Necrosis Factor

Despite the associations between genetic variants within *TNF* and MDD [described in *Section 1.5.1.1.4.*], there have been no reports of genetic variants in *TNF* predicting response to antidepressants. In contrast, studies have reported that higher transcription and protein levels of TNF predict non-responsiveness to antidepressants (Cattaneo et al., 2012; Lanquillon et al., 2000; Tuglu et al., 2003; Eller et al., 2008). For instance, higher baseline TNF protein levels have been found to predict a poor response to both tricyclics (Lanquillon et al., 2000) and SSRIs (Tuglu et al., 2003; Eller et al., 2008).

There are four lines of evidence which support the potential role of TNF in moderating antidepressant response. Firstly. TNF is involved in the expression and functionality of the serotonin transporter, which is the primary pharmacological target of SSRIs (Mossner et al., 1998; Zhu et al., 2006). Consequently, TNF may moderate how antidepressants interact with the serotonin transporter and subsequently affecting response. Secondly, TNF has also been linked to induction of the indoleamine 2,3-dioxygenase (IDO) enzyme which breaks down the precursor to serotonin (Miller et al., 2009). Thus, increased production of TNF may lead to decreased serotonin synthesis, again

moderating the actions of antidepressants (Miller et al., 2009). Thirdly, TNF has also been demonstrated to inhibit hippocampal neurogenesis, which is a further mechanism by which antidepressants are thought to exert their therapeutic effects (Monje et al., 2003; Iosif et al., 2003). Finally, the TNF antagonist infliximab has recently been found to increase responsiveness to antidepressants in those with highest levels of inflammatory markers (Raison et al., 2013). Therefore, TNF is a strong candidate as a predictor of antidepressant response.

1.5.1.2.3 Interleukin-1B

The role of *IL1B* polymorphisms as putative diagnostic biomarkers for MDD has been discussed in *Section 1.5.1.1.1.* However, there is further evidence to show that SNPs with diagnostic properties may also predict treatment response to antidepressants. For example, those homozygous for the C-allele in -511C/T (rs16944) show a less favourable response to the SSRI fluoxetine than T-allele carriers, in a Chinese population (Yu et al., 2003). In contrast, carriers of the -511T/T variant were associated with significantly faster and more pronounced response to the SSRI paroxetine than carriers of the -511C/C allele in a Caucasian population (Tadić et al., 2008). Whereas, there has been no association between -511C/T and response to the noradrenergic and specific serotonergic antidepressant mirtazapine (Tadić et al., 2008).

Further *IL1B* polymorphisms associated with responsiveness to treatment include rs114643 in intron 6 and rs16944 in the promoter region. The G/G genotypes of both of these alleles were found to be significantly associated with non-remission after 6 weeks of antidepressant mono- or poly-therapy with

several antidepressant classes (Baune et al., 2010). However, further analysis of the G/G genotype versus AG/AA groups combined resulted in only rs1143643 polymorphisms being associated with non-remission (Baune et al., 2010).

Only one transcriptomic study in blood has revealed higher levels of *IL1B* mRNA predicting non-responsiveness to antidepressants (Cattaneo et al., 2012). Subsequently, there is a potential use of both genotype and transcriptomic biomarkers within *IL-1B* as predictors of antidepressant response, but it further replication studies are required to draw any firm conclusions.

1.5.1.2.4 Interleukin-11

The Intereukin-11 gene (*IL11*) is located on chromosome 19, consisting of five exons and four introns (McKinley et al., 1992). A SNP in *IL11* was shown to be the best predictive marker of clinical response to escitalopram as part of a genome-wide association study (Uher et al., 2010). Carriers of the A allele in rs1126757 (A/A or A/G) in *IL11* responded better to the SSRI escitalopram, than individuals homozygous for the G allele after 12 weeks of treatment (Uher et al., 2010). IL-11 is a close functional homologue of IL-6, and both of these cytokines are implicated in the inhibition of serotonin production from raphe neurons in the brain stem (see above). This inhibition of serotonin production could offer a possible explanation as to why variants in these genes may affect clinical response to SSRIs. Studies with larger sample sizes and multiple drugs are required to further establish the clinical implications of these SNPs.

1.5.1.3 Antidepressant-induced transcriptional changes and treatment-emergent biomarkers for antidepressant response

As discussed in *Section 1.5.*, the dynamic nature of transcription and protein expression suggests that these biochemical measures may also be used to monitor phenotypic change. Previous reports have revealed changes in the mRNA or protein levels of *IL1B*, *IL6*, macrophage migration inhibitory factor (*MIF*), TNF and IL-10 in response to antidepressant treatment (Lanquillon et al., 2000; Tuglu et al., 2003; Cattaneo et al., 2012). Furthermore, in one study, the magnitude of transcriptional changes in *IL6* after 8 weeks of treatment with antidepressants predicted the magnitude of clinical response after 12 weeks of treatment (Cattaneo et al., 2012). Consequently, transcriptional and proteomic changes to cytokines during antidepressant treatment may be important in mediating antidepressant response. Furthermore, early changes in transcription may be useful in predicting longer-term antidepressant response.

1.6 Conclusion

Despite twin studies suggesting MDD is heritable, research has largely failed to identify specific genes associated with MDD. It has been suggested that gene-environment interactions may contribute to the observed 'missing heritability', with eSLEs perhaps playing a particularly pertinent role in moderating susceptibility to MDD. The long-lasting genome-wide effects of eSLEs however remain unclear, and no GWASs have currently attempted to investigate gene-

environment interactions on a genome-wide scale, which means few novel susceptibility loci have been identified for MDD in response to eSLEs. The search for biomarkers remains a key goal to improve diagnosis and treatment selection for MDD. Evidence suggests that molecular differences in the inflammatory cytokines may act as useful biomarkers for MDD, and for the prediction of antidepressant treatment response.

1.7 Aims

1. To identify novel gene-environment interactions which increase risk to MDD. Using a maternal separation protocol in two inbred strains of mice showing long lasting differences in stress-reactivity in response to separation, we will investigate strain by stress interactions occurring at the transcriptional level in the hypothalamus. We will then form a hypothesis-driven analysis to assess whether genetic polymorphisms in these gene(s) interact with the presence of childhood neglect to predict MDD status in a human clinical cohort.

2. To establish whether transcriptional biomarkers in the inflammatory cytokine pathway can differentiate between controls, MDD patients and bipolar disorder patients. In Chapter 3 we will investigate whether transcriptional differences within the inflammatory cytokine pathway (using whole blood) can be used to differentiate MDD, bipolar disorder patients and controls. This is in an attempt to identify disorder-specific biomarkers which may reduce clinical misdiagnosis, a problem discussed in *Section 1.4.*

3. To establish whether transcriptional and epigenetic biomarkers in the inflammatory cytokine pathway can be used as predictors of clinical antidepressant response. In chapter 4 we attempt to identify transcriptional predictors of antidepressant response in the inflammatory cytokine pathway in hopes of tackling the clinical problem of ineffective treatment, discusses in *Section 1.4*. Based on these results, we select further genes of interest to investigate at the DNA methylation level, in an attempt to identify epigenetic predictors of antidepressant response.

4. To investigate whether antidepressant-induced transcriptional changes in the inflammatory cytokine pathway correspond to clinical response. Finally, chapter 5 investigates the effects of an antidepressant on the transcription of cytokine genes and establishes whether molecular changes relate to clinical antidepressant response. Results from this study aims to identify potential molecular targets which may be important in moderating antidepressant efficacy, and aims to identify potential treatment-emergent transcriptomic biomarkers.

Chapter 2

Investigating gene-environment interactions for major depressive disorder based on whole genome transcriptomic changes in mouse brain in response to early life stress

Figure 2.0: Photographs of DBA/2J (top) and C57BL/6J (bottom) inbred mouse strains. Images adapted from: http://jaxmice.jax.org/strain/ .

2.1 Introduction

MDD is a heterogeneous disorder with numerous putative aetiological pathways and environmental and genetic risk factors (Lesch, 2004). Early stressful life events (eSLEs), such as childhood neglect and abuse, represent one environmental risk factor known to increase an individual's susceptibility to MDD (Kaufman et al., 2001). Studies on rodent models have also shown that early separation of pups from their mothers, likened to childhood neglect in humans, alters the epigenome, the transcriptome, neurobiological circuitry and corticosterone reactivity, resulting in long-lasting physiological, and behavioural changes thought to parallel those found amongst MDD patients (Meaney, 2001; Kember et al, 2012).

Although eSLEs increase risk for MDD in some individuals, they do not increase risk for MDD in everyone. One attractive theory to explain this inter-individual variability in MDD risk on exposure to eSLEs is that MDD may result from the interaction between genetic susceptibility loci and eSLEs (Keers et al., 2011). For instance, both childhood neglect and abuse have been found to increase an individual's susceptibility for MDD in later life, but these events also show interactions with previously identified susceptibility loci for MDD, such as *5-HTTLPR*, to moderate risk for MDD (Uher et al., 2011, Fisher et al., 2013). Based on the promising results from candidate gene-environment interaction studies [see *Section 1.1.6* for more information], there is now an urgent need to explore whether other susceptibility loci across the genome may exist which interact with specific eSLEs to predispose to MDD.

The largest genome-wide association study for MDD to-date, however, has arguably been underpowered and was unable to identify novel genes robustly

associated with MDD (Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium, 2013). It has subsequently been argued that even greater power will be required to detect gene-environment interactions on a genome-wide scale, after the burden of multiple testing correction (Duncan & Keller, 2011). Consequently, despite the promising findings detailed in candidate gene-environment interaction studies, progress in the field is limited and genome-wide studies remain a key goal for the future.

One way of prioritizing the investigation of novel MDD susceptibility loci in humans in a hypothesis-free manner stems from the utilization of animal models. For instance, a study by Malki et al. (2010) exploring the moderating effects of genetic variants on antidepressant response in a human clinical cohort, prioritized their investigation by selecting genes showing differential expression changes in the mouse brain in response to antidepressant treatment. Subsequently, similar methods could be employed to identify susceptibility loci for MDD in humans, by prioritizing genes which show expression changes in the mouse brain in response to eSLEs.

A recent study by our group, exploring the effect of an eSLE in two genetically distinct inbred mouse strains, revealed strain-specific molecular, behavioural, and hormonal changes in response to maternal separation (Kember et al., 2012). Of particular significance was the identification of a strain-specific exacerbation in stress reactivity in adult mice that had undergone a maternal separation; with C57BL/6J but not DBA/2J mice showing increased corticosterone release on exposure to a later life stressor (Kember et al., 2012). Elevated corticosterone reactivity and alterations to the hypothalamic-pituitary-adrenal (HPA) axis are among the most reliable physiological biomarkers for

MDD to-date (Hellhammera et al., 2009). Consequently, these inbred strains may be modelling the effects of eSLEs in eSLE/MDD-vulnerable (C57BL/6J) individuals and eSLE/MDD-resistant (DBA/2J) individuals. Thus, examining strain-specific molecular differences in response to separation may provide an insight into how eSLEs increase susceptibility to MDD in humans.

In the current study we investigate strain by stress (separation) interactions at the level of the transcriptome, which represents a functional molecular output of gene-environment interactions. We measure gene expression in the hypothalamus (the control centre of the HPA-axis and stress response) in C57BL/6J and DBA/2J inbred mouse strains, and attempt to identify transcripts showing differential expression changes in response to maternal separation. We then use this to prioritise the investigation of potentially novel susceptibility loci interacting with childhood neglect to predict MDD case/control status in a human clinical cohort.

2.2 Methods

2.2.1 Animals

C57BL/6J and DBA/2J mice were bred in the Biological Services Unit at the Institute of Psychiatry, Kings College London using original stocks [respective stock numbers: 000664, 000671] purchased from The Jackson Laboratory (Bar Harbor, ME, USA). DBA/2J and C57BL/6J strains were selected as these represent members of a priority list based on the most well-characterized, commonly used strains for gene manipulation and crosses (Mouse Phenome Project, http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home).

Mice were housed in standard cages measuring 30.5×13×11 cm, kept at an ambient temperature (21±2°C) and light (light/dark cycle with white lights on from 08:00 to 20:00). Food for breeders (Rat and Mouse No. 3 diet, Special Diet Services, Essex, UK), food for test mice (Rat and Mouse No. 1 diet) and tap water was available *ad libitum*. Sawdust (Litaspen premium) and nesting materials (Sizzlenest, Datsand, Manchester, U.K.) in each cage were changed once every two weeks, but never on the day before or the day of testing to minimize the disruptive effect of cage cleaning on behavior. At postnatal day one, litters were reduced to 6 pups to standardize litter sizes. All housing and experimental procedures were performed in compliance with the UK Home Office Animals Scientific Procedures Act 1986.

2.2.2 Modelling childhood neglect

To model childhood neglect, a maternal separation protocol was used. Males were paired with female breeders for 2 weeks and then removed. Litters of each strain were randomly allocated to control or maternal separation groups. For the litters in the maternal separation group, the mother was removed from the litter on postnatal day 9 for 24 hours and returned to the housing room, leaving the pups in a procedure room. The cages containing the litters were placed on a heating pad and kept in a procedure room in order to maximize separation from their mother. After 24 hours, the dam was returned to the litter and the cage returned to the housing room. Control group litters were not disturbed and remained in the housing room with their mothers until they were weaned. Mice were weaned aged 5 weeks and two pups within each litter were randomly

assigned to one of three groups; test-naïve adolescent group (culled at 5 weeks), test-naïve adults (culled at 14-15 weeks) and test adults (tested at 11-12 weeks, culled at 14-15 weeks). The groups of adult mice were transferred at approximately 9 weeks of age to a separate housing and test facility and pair housed with a same sex sibling. All mice were allowed to habituate for 2 weeks before being either culled or undergoing a battery of behavioural tests and then culled. Culling was carried out using cervical dislocation and the hypothalamus and hippocampi were dissected. For the gene expression experiments, only adult male mice were used and there was a total of 23 control mice (n=10 C57BL/6J, n=13 DBA/2J) and 19 separated mice (n=9 C57BL/6J, n=10 DBA/2J). In order to reduce any effects behavioural testing may have on gene expression, the majority of mice used in gene expression experiments were behaviourally naïve except for three of the DBA/2J mice, which was corrected for when dealing with batch effects.

2.2.3 Whole Genome Transcriptomics

2.2.3.1 Ribonucleic acid

Ribonucleic acid (RNA) is a chain of ribonucleotides (Berg et al., 2002). RNA primarily consists of three subtypes: ribosomal RNA (rRNA), transfer RNA (tRNA) and messenger RNA (mRNA) (Berg et al., 2002). The relative differences in total RNA extracted from biological material experimentally is usually attributed to quantitative differences in mRNA levels, which represents the molecular measure of gene expression, and codes for the sequence of amino acids in a polypeptide (Cooper & Haussman, 2004).

RNA has a short half-life and degrades quickly as result of the actions of enzymes which break down RNA (RNAases). Consequently, extracted RNA must be kept at low temperatures to inhibit the actions of these enzymes, undergo as few freeze-thaw cycles as possible, and be checked for degradation prior to gene expression experiments (Holzmann et al., 2008).

2.2.3.2 RNA Extraction

The Qiagen AllPrep DNA/RNA kit (Crawley, UK) was used to extract RNA and DNA, using the manufacturer's standard protocol. Briefly, hypothalami were homogenized using disposable microcentrifuge tube tissue grinders (Anachem, UK). Subsequently, the homogenised tissue was spun through porous columns which trap nucleic acids. Nucleic acids are then washed and eluted, culminating in separate purified DNA and RNA elutions (see Figure 2.1).

2.2.3.3 RNA Quantification

RNA was quantified and checked for purity using the Nanodrop ND100 (Thermoscientific, Wilmington, Delaware, USA). The concentration of each RNA sample was assessed by determining the absorbance of ultra violet light at 260nm in a spectrophotometer using a quartz cuvette. The ratio of spectrophotometer readings at 260nm and 280nm was also used to estimate the purity of RNA, with 260/280 ratios between 1.8 and 2.1 considered good purity, and free of protein contamination. All RNA samples in the current study were of good purity.

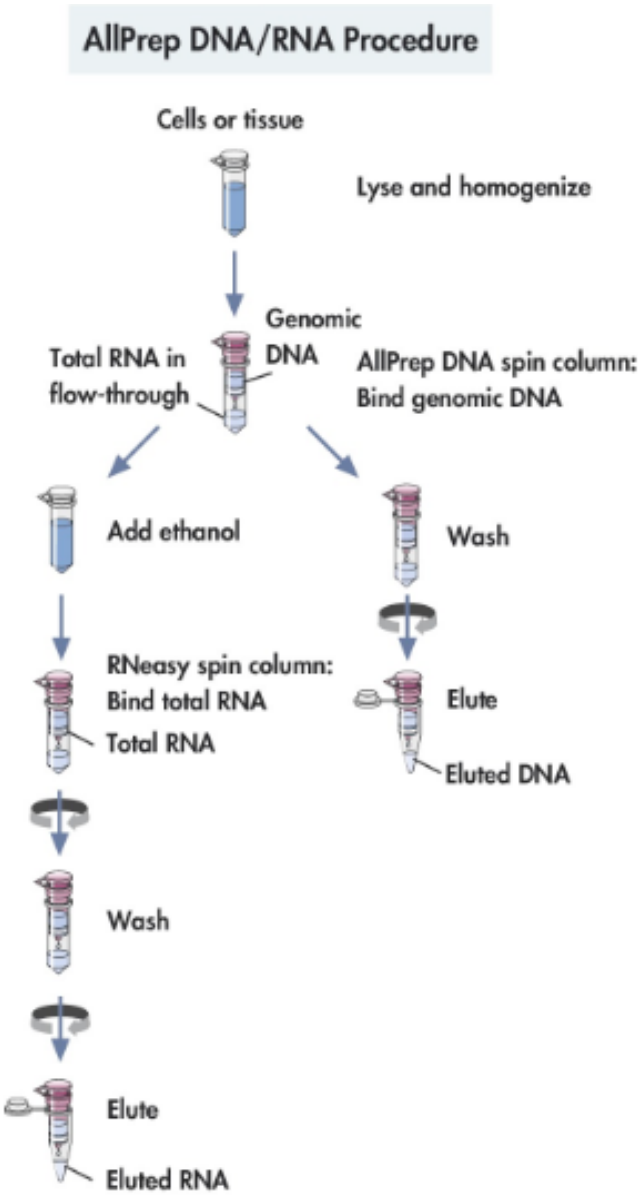


Figure 2.1: Key steps involved in RNA and DNA extraction using the Qiagen AllPrep DNA/RNA kit. Image adapted from: http://www.qiagen.com/Products/Catalog/Sample-Technologies/RNA-Sample-Technologies/DNA-RNA-Protein/AllPrep-DNARNA-Mini-Kit#productdetails .

2.2.3.4. RNA Quality

As mentioned in *Section 2.2.3.1,* RNA has a short half-life and degrades quickly which can affect its suitability for use in gene expression experiments. Subsequently, it is necessary to assess whether extracted RNA samples all show relatively low levels of degradation. To assess RNA quality we used the Agilent 2100 Bioanalyzer (Agilent Technologies, Berkshire, UK). This system utilises a multi-well chip in which 12 RNA samples can be included per run. Briefly, the procedure involves using an electric current to drive RNA intercalated with a fluorescent dye towards electrodes. A constant mass-to-charge ratio means that molecules are separated by size with smaller fragments migrating faster than larger, heavier fragments. Two very large rRNA molecules (18S and 28S) form two distinctive peaks on generated electropherograms (see Figure 2.2). The sharpness of the 18S and 28S peaks and the ratios between these peaks are incorporated along with other information to form a quantitative measure of RNA integrity, a RNA integrity Number or "RIN". RNA samples generating RINs between 7 and 10 are usually considered good quality. All RNA samples used in this study had RINs of 8±1.

2.2.3.5. Whole Genome Amplification

100ng of extracted RNA was processed with the Ambion WT expression kit and underwent whole genome amplification using the standard manufacturer protocols. The amplification process consisted of 10 steps: (i) first strand cDNA synthesis, (ii) second strand cDNA synthesis, (iii) cRNA synthesis, (iv) cRNA purification, (v) assessment of cRNA yield and size distribution, (vi) synthesis of second cycle DNA, (vii) hydrolysis, (viii) purification of second cycle DNA, (xi)

assess cDNA yield and size distribution, (x) fragment and label the single-stranded cDNA.

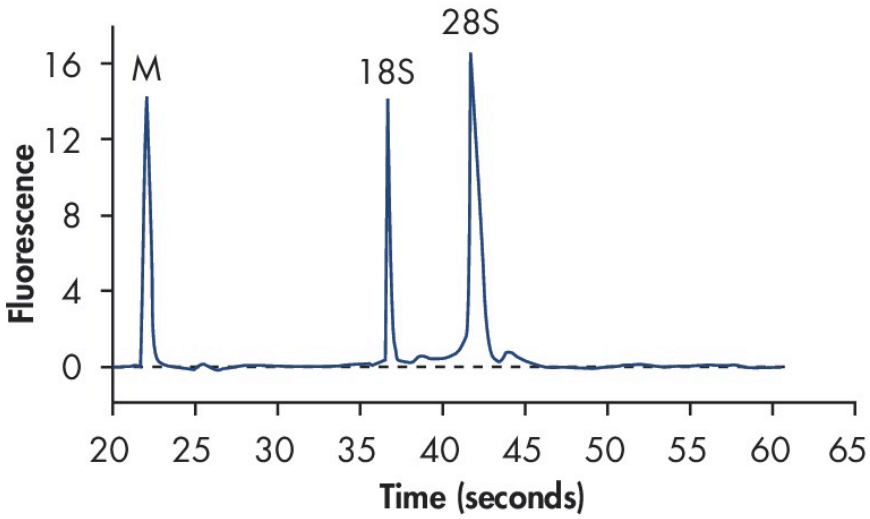


Figure 2.2: An electropherogram trace for non-degraded RNA. Note the high and sharp florescent signals generated for M (marker), and 18S and 28S ribosomal RNA fragments.

2.2.3.6. Affymetrix Gene Chip 1.0 ST arrays

Affymetrix Gene Chip 1.0 ST arrays (Affymetrix, High Wycombe, UK) were used to measure genome-wide expression and contain probes covering all of the 28,853 genes within the Mus musculus genome. Using the Affymetrix Gene Chip Hybridisation Kit, 5.5 ug of synthesized single-stranded cDNA was fragmented, labelled and then hybridized to the arrays. Arrays were then washed and stained and then scanned in the Affymetrix Gene Chip 3000 machine. AGCC scan control software was used to generate cel files.

2.2.3.7. Array Pre-processing and Normalisation

Data normalisation was performed using the Robust Multiarray Average (RMA) package available at www.bioconductor.org for use in R (http://www.R-project.org). Briefly, pre-processing of the array data using RMA involves three key steps: (i) RMA convolution; which implements background adjustment during which perfect match values are corrected array-by-array using a global model of the distribution of probe intensities; (ii) Quantile normalisation; this process impose the same empirical distribution of intensities to each array; (iii) Summarization; the process combines the multiple probe intensities for each probeset to produce an expression value (Irizarry et al., 2003). The final output is log-transformed RMA values signifying probe intensity summaries for each gene.

2.2.4 Human Genetic Association Study

2.2.4.1. Participants

Subjects with recurrent MDD and healthy controls were drawn from two sites within the UK, Cardiff and London, as part of the Depression Case–Control (DeCC) study (see Cohen- Woods et al., 2009). The DeCC study was approved by the local ethics committees at each site and all participants provided written informed consent.

All participants in DeCC were over the age of 18, and of White European parentage. Subjects were identified through psychiatric clinics, hospitals, general medical practitioner surgeries, and media advertisements. MDD patients must have experienced at least two major depressive episodes of

at least moderate severity and separated by two or more months of remission, as defined by DSM-IV (American Psychiatric Association, 2000) and/or ICD-10 (World Health Organisation, 1993). Major depression diagnosis in recruited patients was confirmed at interview using the Schedules for Clinical Assessment in Neuropsychiatry (SCAN; Wing et al., 1990). Exclusion criteria were history of mania or hypomania, mood-incongruent psychosis, and a first or second- degree relative with bipolar or psychotic disorder. MDD patients were also excluded if their depression had only occurred secondary to physical illness, medication, or substance or alcohol misuse, if they were intravenous drug users with a lifetime diagnosis of dependency, or if they were related to an individual already included in the study group.

Control subjects were recruited through UK general medical practices and excluded if they had a personal or first-degree relative with a history of any psychiatric disorder. They were screened for lifetime absence of psychiatric disorders using a screening method adapted from the Past History Schedule (McGuffin et al., 1986). Control subjects were also excluded if they scored 10 or above on the Beck Depression Inventory (BDI) (Beck & Steer, 1984), or failed to return consent or cheek swabs.

2.2.4.2. Measures of Early Life Stress

Self-reported information on emotional abuse, physical abuse, sexual abuse, emotional neglect (EN) and physical neglect (PN) during childhood were captured using the Childhood Trauma Questionnaire (CTQ; Bernstein et al., 2003). In total we had CTQ ratings for 197 recurrent MDD cases and 286

screened controls. For the purposes of this study we only utilise information related to neglect (i.e. EN and PN), as these measures relate more strongly to the maternal separation model in which the pup is both physically and emotionally absent from their mother. Subscale scores of the CTQ were coded as none (0), mild (1), moderate (2) and severe (3) in accordance with the manual (Bernstein et al., 2003). As in Fisher et al., (2013) moderate (2) and severe (3) categories for each maltreatment type were collapsed into one group due to the small numbers of participants in the 'severe' category in DeCC.

2.2.4.3. Genome-wide genotyping

Genomic DNA was extracted from bloods and cheek swabs collected as described previously (Freeman et al., 2003). DNA samples were then sent to the Centre National de Genotypage (Evry Cedex, France) and were genotyped using the Illumina Human610-Quad bead chip (Illumina, Inc., San Diego, CA, USA). Genotype data for single nucleotide polymorphisms (SNPs) under investigation in this study were extracted using PLINK (Purcell et al., 2007).

2.2.5. Statistical Analysis

Testing for significance in our expression data was achieved using a univariate linear regression, which incorporated main effects (strain, stress), nuisance factors (array batch effects) and our interaction effect (stress x strain interaction). The False Discovery Rate (FDR) method of multiple testing correction was applied, and q-values of $q<0.1$ were considered true discoveries. Ingenuity Pathway analysis was performed for all probes producing p-values of $p<0.01$ in order to

identify any gene networks affected as part of strain x stress interactions. For our top stress x strain interaction we further investigated this gene in our human cohort. We extracted SNPs 30KB upstream and 10KB downstream of the gene. The 'genhw' package in STATA 11.0 (StataCorp, Texas, USA) was used to assess whether genotype frequencies diverged from the Hardy-Weinberg Equilibrium. We then assessed whether either PN or EN showed an interaction with extracted SNPs to predict MDD status. The main effects of the SNP, the main effect of neglect (either EN/PN) and interactions between childhood neglect and the SNP, on the presence/absence of recurrent MDD was examined using a generalized linear models with a binomial distribution and identity link function specified (Wacholder, 1986), and was adjusted for the effects of gender, as in Fisher et al., 2013. For any SNP which generated a significant interaction in our model ($p<0.05$), we further tested whether one allele might be dominant in driving the interaction. We achieved this by dichotomizing genotypes into those carrying at least one copy of the major allele (1/0), and repeating our generalized linear model. We then dichotomized genotypes into those carrying at least one copy of the minor allele (1/0) and repeated the linear model. If one allele shows a stronger interaction with neglect, we assume this is the dominant allele driving the interaction.

2.6. Results

2.6.1. Whole Genome Transcriptomics

Univariate linear regressions revealed no significant stress x strain interactions at the transcriptomic level after multiple testing correction, i.e. all transcripts

generated q>0.1. The results from the top eight stress x strain interactions are given in Table 2.1 and graphically presented in Figure 2.3. The top interaction was found in the probe 10500345, which relates to the transcript telomerase RNA component (*Terc*). The top associated network based on pathway analysis was 'Organ Morphology, Reproductive System Development and Function, Cardiac Arrythmia', which also featured *Terc*, see Figure 2.4. Subsequent *in silico* analysis confirmed that there is a SNP downstream (<10KB) of the *Terc* gene (MRS1058401), which differs between C57BL/6J (GG carriers) and DBA/2J mice (TT carriers).

Probe Identifier	Gene Symbol	Chromosome	p-value
10500345	Terc	3	1.70E-05
10505879	Ifna7	9	1.75E-04
10493494	Efna3	1	1.95E-04
10414485	Olfr265	14	2.22E-04
10498795	Zfp108	7	3.00E-04
10551393	Akt2	10	4.64E-04
10500103	Gabpb2	3	4.75E-04
10508089	Mrps15	4	5.10E-04

Table 2.1 Table displaying the eight most significant probes showing stress x strain interactions at the transcriptomic level in the hypothalamus, based on results from univariate linear regressions. Information listed includes the probe identifier on the Affymetrix Gene Chip 1.0 ST array, the corresponding gene, its chromosomal location, and p-value.

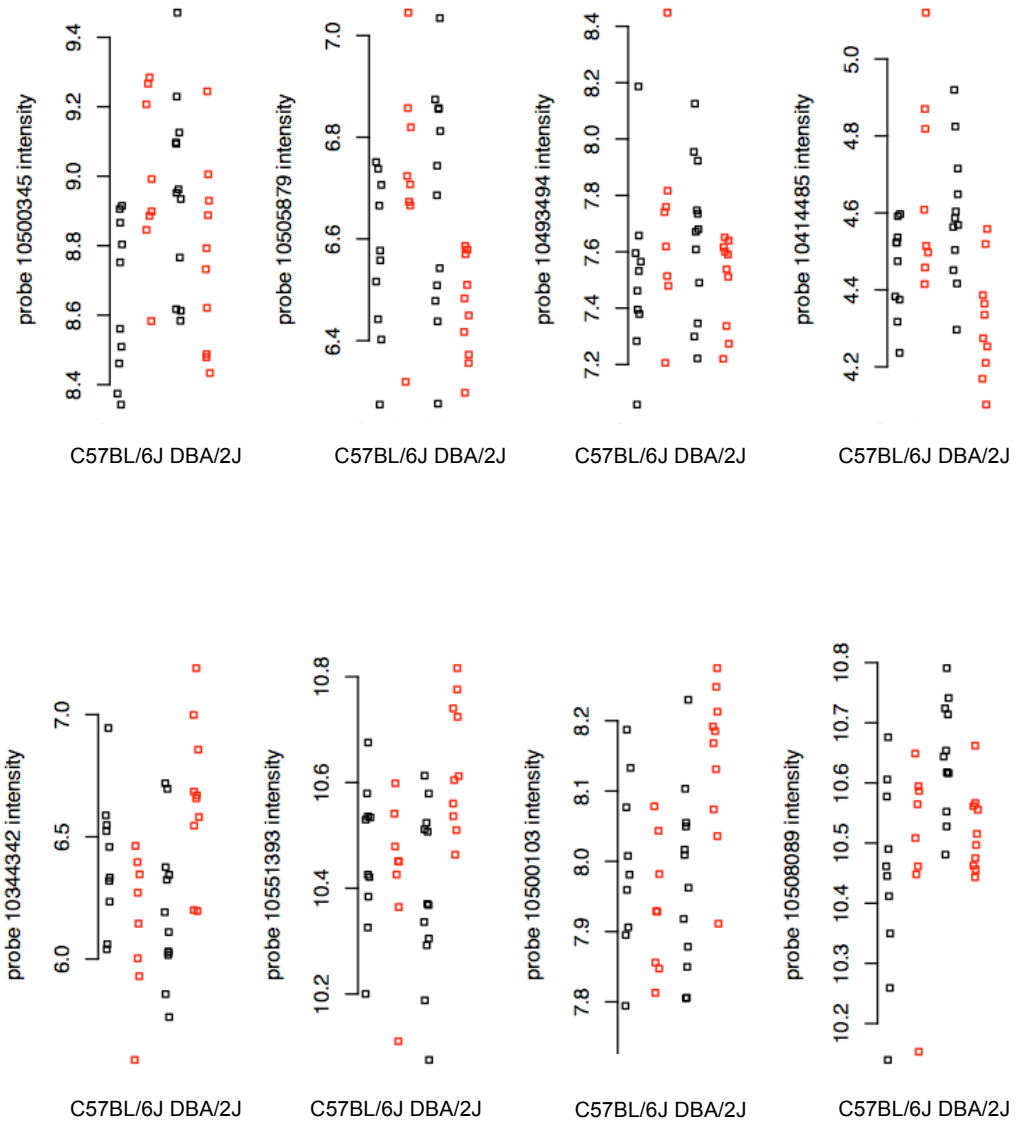


Figure 2.3: Strip charts showing the distribution of probe intensities (RMA values) for C57BL/6J mice shown on the left of each chart, and DBA/2J shown on the right of each chart. Results from controls are shown in black and results from maternally separated mice are shown in red.

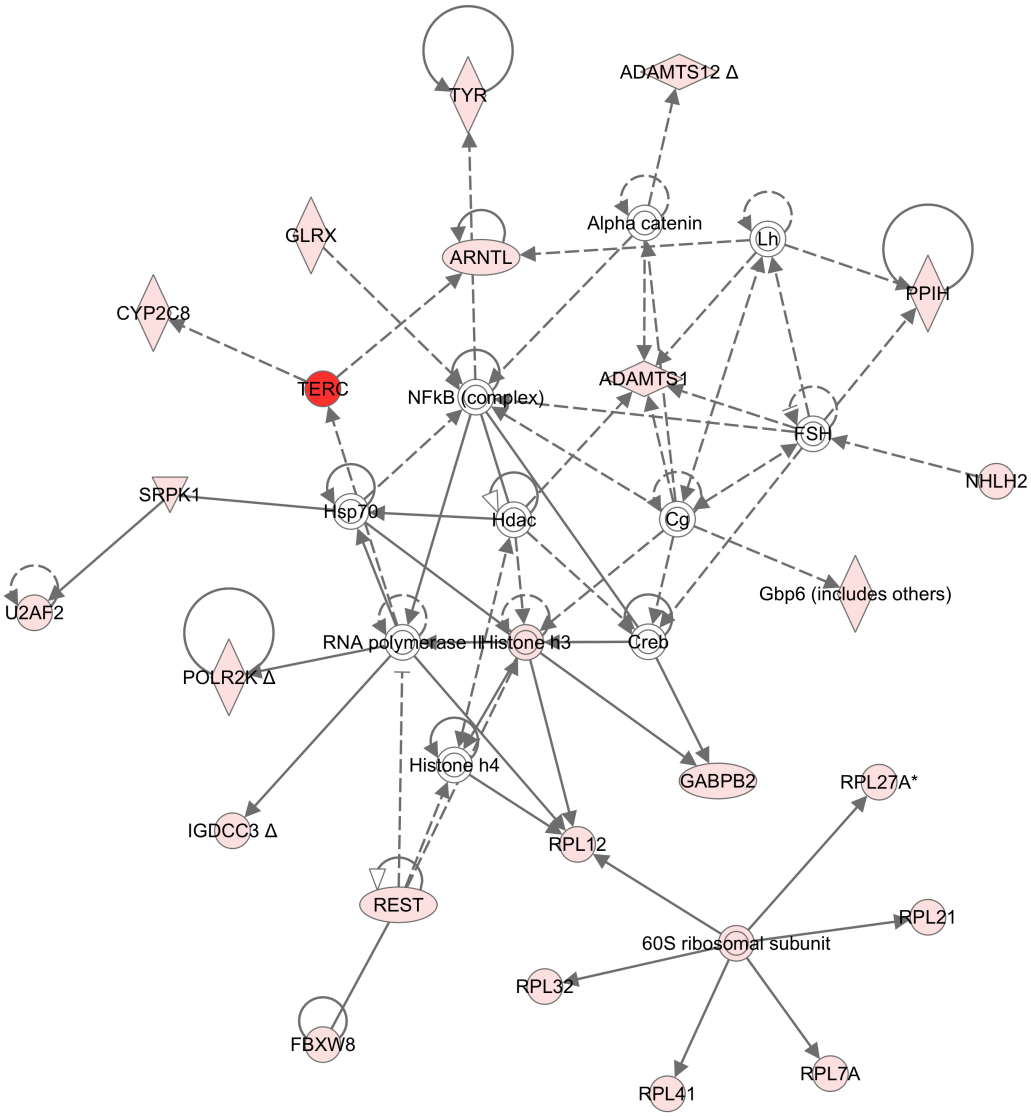


Figure 2.4: Results from Ingenuity Pathway Analysis. Figure depicts genes involved in the "Organ Morphology, Reproductive System Development and Function, Cardiac Arrythmia" network which was the top gene network showing expression differences as part of a stress by strain interaction in the hypothalamus in response to maternal separation. Results were based on inputting 239 transcripts which produced uncorrected p-values of $p<0.01$ from strain by stress analyses into Ingenuity Pathway Analysis. The transcript showing the most significant stress by strain interaction, *Terc*, is shown in darker red.

2.6.2. Human Genetic Association

We extracted genotype information using PLINK (Purcell et al., 2007) for all SNPs located -30KB and +10KB of *TERC* (chr3:170,965,092-170,965,542; Genome Reference Consortium GRCh37/USCS version hg19). Three SNPs within this region were present on the genotyping platform with the following genotype frequencies: rs11718668 [CC (n=256), CT (n=189), TT (n=38)], rs1881984 [AA (n=230), AG (n=204), GG (n=49)], rs10936599 [CC (n=294); CT (n=158); TT (n=31)]. None of the genotypes deviated from Hardy-Weinberg equilibrium. Results revealed that the SNP furthest downstream, rs10936599, significantly interacted with severity of emotional neglect to predict MDD status, see Table 2.2. rs10936599 consists of a major allele C, and minor allele T. We found the dominant effect to be on the C-allele, with those carrying at least one copy of the C-allele showing strongest interactions with emotional neglect to predict MDD status [R.D. =-0.063; 95% C.I. = 0.017 - 0.108; p = 0.007], rather than those carrying at least one copy of the T-allele [R.D. = 0.061; 95% C.I. = -0.001 - 0.124; p=0.055].

Interaction	RD	95% CI		p
rs11718668 x EN	0.015	-0.060	0.091	0.690
rs10936599 x EN	0.078	-0.149	0.007	**0.029***
rs1881984 x EN	0.039	-0.032	0.111	0.280
rs11718668 x PN	-0.001	-0.077	0.075	0.986
rs10936599 x PN	0.037	-0.022	0.095	0.218
rs1881984 x PN	0.011	-0.060	0.082	0.767

Table 2.2 Results from linear regressions investigating whether interactions existed between each of our three SNPs (rs11718668, rs10936599, rs1881984) and either emotional neglect (EN) or physical neglect (PN) to predict MDD case/control status. Results detailed include risk difference (RD), 95% confidence intervals (95% CI) and p-values (p). Significant interactions ($p<0.05$) are highlighted with a *.

2.7. Discussion

Gene-environment interactions represent a potentially important mechanism through which eSLEs increase some individuals' susceptibility to MDD (Nugent et al., 2011). Currently, insufficient power is available to detect novel gene-environment interactions for MDD on a genome-wide scale, however methods can be employed to narrow down the search (Duncan & Keller, 2011; Malki et al., 2010). The current study utilised a maternal separation procedure in two inbred strains of mice, which exhibit different stress-reactivity profiles in adulthood in response to separation (see Kember et al., 2012). The project aimed to model childhood neglect and investigate whether stress by strain

interactions present at the level of the transcriptome could be used to prioritise the search for gene-environment interactions for MDD in humans.

Our study revealed no stress by strain interactions which remained significant after multiple testing correction (see Table 2.1). However, our top interaction was found in the transcript telomerase RNA component (*Terc*) (see Table 2.1 and Figure 2.3), which is also featured in our top gene network (see Figure 2.4). *Terc* codes for the RNA component of the telomerase enzyme, which is a ribonucleoprotein polymerase that maintains telomere ends (Marrone & Dokal, 2007). Specifically, Terc provides the template for creating the repeated sequence of DNA, TTAGGG, that telomerase adds to the ends of chromosomes (Shalev, 2012). Telomere shortening has been associated with aging and increased numbers of cell divisions, and mutations in the *TERC* gene have previously been associated with the aging-like disease dyskeratosis congenital (Vulliamy et al., 2011). Recent research has also reported shorter telomeres amongst adults who have experienced emotional and physical neglect in childhood (Tyrka et al., 2010), adults who have experienced childhood trauma (O'Donovan et al., 2011), and in children who have experienced domestic violence (Shalev et al., 2013). Furthermore, shorter telomeres have been reported amongst patients with psychiatric disorders, and particularly amongst patients with mood disorders (Simon et al., 2006; Hartmann et al., 2010; Elvsashagen et al., 2011; Wikgren et al., 2012).

There are numerous theories about the processes which may be involved in accelerated telomere shortening or 'telomere erosion' in response to eSLEs, such as neglect. Theories include immune and endocrine activation, oxidative stress and mitochondrial dysfunction (Shalev, 2012). However, most current

theories converge around the idea that a disturbed HPA axis and heightened cortisol levels, are linked to greater 'cellular aging' and telomere erosion (Wickgren et al., 2012). In this study, we provide the first evidence that an eSLE, provoking strain-specific alterations in HPA-reactivity, is associated with differential transcriptional rates for the telomerase coding gene *Terc* in the hypothalamus. Alterations to the expression of *Terc* in response to stress provides a potential mechanism through which eSLEs evoke enduring alterations to telomere length.

Based on these findings, we further tested whether SNPs within *TERC* (or 30KB upstream/10KB downstream of *TERC*) interacted with severity of physical or emotional neglect (human phenotypes most closely analogous to maternal separation) to predict MDD status in a human clinical MDD case-control cohort. Results revealed that a SNP downstream of *TERC*, rs10936599, located in the exon of a neighbouring gene coding for a BTB/POZ and zinc finger transcription factor, myoneurin (MYNN), interacted specifically with severity of emotional neglect to predict MDD case/control status. The presence of at least one copy of the major C-allele was found to most strongly interact with emotional neglect to predict MDD case/control status. Interestingly, rs10936599 has been identified as the strongest genetic predictor of telomere length as part of a recent GWAS (Codd et al., 2013), with the major C-allele being associated with longer telomeres, relative to the minor T-allele (Jones et al., 2012). rs10936599 is also in strong linkage disequilibrium (LD) with a known functional SNP within the *TERC* gene, rs2293607, known to affect *TERC* mRNA expression and telomere length (Jones et al., 2012). Consequently, our results may suggest that those with a genetic predisposition to longer telomeres (C-allele carriers) are

particularly vulnerable to the effects of childhood neglect. However, the exact mechanisms through which *TERC* expression and telomere length might impact upon the pathophysiology of MDD remains unclear.

Despite the promising results detailed here, there are numerous limitations to the current study. Firstly, the animal component of the study utilised a small number of mice, and we were unable to detect any significant strain by stress interactions after multiple testing correction. Therefore, the effects neglect may have on the expression of *TERC* in the mouse brain need to be replicated in a larger sample to confirm it is a true effect. Secondly, we liken maternal separation in this study to childhood neglect because it involves the absence of physical and emotional support from the primary carer during early life. However, this interpretation is likely over-simplified. The maternal separation procedure employed here models an acute form of 'neglect', whereas human childhood neglect is probably more chronic in nature, occurring on repeated occasions, with differing severities. Consequently, a single separation protocol may not accurately model the complexities associated with childhood neglect. Thirdly, measures of telomerase activity and telomere length would be beneficial to confirm that the observed differences in *TERC* expression correspond to functional differences in enzyme activity and telomere length. Furthermore, the animal component of the study observes the effects of separation cross-sectionally, and the human component assesses neglect retrospectively. A future study design should investigate the effects of separation in mouse in a longitudinal manner and observe whether cumulative later life stressors further have an effect on *Terc* expression and telomere length in the hypothalamus. Similarly, longitudinal studies collecting information about eSLEs

(e.g. as reported in Shalev et al., 2013) may limit the effects of any retrospective reporting bias present in this study. Additionally, our human sample is still relatively small and the interaction between rs10936599 and emotional neglect requires replication. Finally, it would be useful to test whether rs10936599 or rs2293607 (SNP in LD with rs10936599) interacts with childhood neglect in humans to differentially predict *TERC* expression levels and telomere length.

In conclusion, our results are the first to reveal potentially important interactions between different genetic backgrounds and early neglect on the expression of the telomerase gene *Terc*. Furthermore, a SNP (rs10936599) downstream of *TERC* previously found as the strongest genetic predictor of telomere length, significantly interacted with severity of early emotional neglect to predict MDD status in a human clinical case-control cohort. If replicated, this could provide a potential aetiological pathway between childhood neglect and MDD, and may allow for the creation of preventative medical interventions.

Chapter 3

Identifying disorder-specific transcriptomic biomarkers in the inflammatory cytokine pathway for major depressive disorder and bipolar disorder

Figure 3.0: 'Depression'. Image adapted from:

http://tribune.com.pk/story/450827/health-hazards-doctors-ignore-most-depression-symptoms/ .

3.1 Introduction

The term 'mood disorder' refers to a category of psychiatric illnesses that are characterized by a pathological distortion of emotional mood (Jones et al., 2002). Mood disorders represent the most common form of severe adult-onset psychiatric disorder and are predicted to be the second most common cause of morbidity by 2020 (Craddock & Forty, 2006; Murray & Lopez, 1996). They consist of two aetiologically related (McGuffin et al., 2003) but distinctly treated psychiatric illnesses (Nemeroff & Owens, 2002), MDD and bipolar disorder (BPD). Both MDD and BPD are clinically characterized by episodes of depression (e.g. lowered mood, loss of interest or pleasure, loss of energy); with BPD also consisting of episodes of mania or hypomania (e.g. expanded self-esteem, increased distractibility, talkativeness; World Health Organisation, 1992; American Psychiatric Association, 2000).

Despite the establishment of clinical diagnostic criteria for MDD and BPD, the heterogeneous nature of these disorders, the similarities they share in their clinical presentation, and the absence of specific biomarkers, means there are relatively high rates of misdiagnosis (Kendell, 1976; Farmer & McGuffin, 1989). BPD is often misdiagnosed in the first instance (Hirschfeld et al., 2003), and an estimated 5.7 years on average is required for the correct diagnosis (Morselli & Elgie, 2003). Most frequently, BPD is misdiagnosed as MDD due to their overlapping symptomology, the often later onset of mania, and more frequent occurrence of depressive episodes in BPD patients (Lewis et al., 2003; Perlis, 2005). Misdiagnosis may be particularly high when BPD patients present symptoms indicative of a clinically significant depressive episode but are premorbid for manic symptoms, or have failed to recognize previous manic

states. Misdiagnosis, and therefore incorrect treatment of BPD with monotherapy antidepressant treatment, increases the risk of antidepressant induced mania (Wehr & Goodwin, 1979; Altshuler, et al., 1995) and "cycle acceleration" (an increased frequency of episodes) (Perlis, 2005); both of which can have damaging effects on disease prognosis. Consequently, the establishment of biomarkers specific to each disorder remains a key goal, so that the correct diagnosis and treatment can be obtained for a patient from the outset.

The clear need for an objective, empirical method of diagnosis has led to genome-wide association studies (GWASs) attempting to identify genes associated with MDD and BPD. However, despite twin studies suggesting mood disorders are moderately heritable, GWASs have largely been unsuccessful in identifying genes robustly associated with MDD (McGuffin et al., 2003; Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium, 2012), with only recent reports from very large-scale studies finding genes potentially being associated with BPD (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011). In addition to genetic background, it has been established that environmental factors, such as stressful life events, can also increase a person's susceptibility to developing a mood disorder, and precipitate mood disorder episodes (Kendler et al., 1999; Hosang et al., 2012). Subsequently, it has been proposed that a lack of findings from GWASs might relate to the more salient presence of gene-environment interactions (Keers & Uher, 2012), as supported by studies in the field (e.g. Caspi et al., 2003; Kim et al., 2007; Bradley et al., 2008; Fisher et al., 2013; see *Section 1.1.6*). Therefore, it may be the interface between genes and environment that contains the most valuable biomarker information about mood disorders, as opposed to genotype

alone. Thus, focusing efforts on identifying biomarkers at the level of the transcriptome, which represents the functional molecular output of gene-environment interactions, might yield more fruitful results.

Cytokines are a group of cell-signaling molecules which, in the periphery, aid inflammatory processes and the immune system to form co-ordinated responses to infection (Dantzer et al., 2008). Cytokines are also expressed centrally and have effects on the brain, influencing neurotransmitter systems, neuroendocrine function and neural plasticity, and converging evidence suggests they may play an important role in the pathophysiology of mood disorders (Miller et al., 2009). Furthermore, cytokines can cross the blood-brain barrier (Banks et al., 1995), so peripheral cytokines may represent a potentially useful biomarker resource relating to mood disorders. Indeed, both protein and transcriptomic studies performed in blood have revealed differences in the expression of cytokines such as interleukin-6, tumor necrosis factor and interleukin-1β amongst MDD patients relative to controls (Tsao et al., 2006; Cattaneo et al., 2012; Hiles et al., 2012; Liu et al., 2012). Similarly, the transcription of cytokines has been found to differentiate between BPD patients and controls (Padmos et al., 2008). However, no studies have yet investigated whether disorder-specific transcriptional differences exist within the inflammatory cytokine pathway, or whether this could be harnessed as clinical diagnostic aids to differentiate between MDD and BPD patients.

The current study aims to identify transcriptomic biomarkers in the inflammatory cytokine pathway which could be used to distinguish between controls subjects, MDD patients and BPD patients. We achieve this using RNA extracted from whole blood and examine an extensive set of inflammatory-

related transcripts including genes coding for: interleukins and interleukin receptors, chemokines and chemokine receptors, the tumour necrosis factor cytokine family and receptors, and other inflammatory regulators. We initially test for differences in a discovery cohort (total n=90), and then attempt to replicate any findings from our discovery cohort in a pseudo-independent validation cohort (total n=36).

3.2 Methods

3.2.1 Clinical Samples

Patient samples used in this study were collected from two methodologically similar studies, the Bipolar Association Case–Control Study (BACCS) (Cohen-Woods et al., 2010) and the Genome-based Therapeutic Drugs for Depression Project (GENDEP) (Uher et al., 2010).

3.2.1.1 Bipolar Disorder Patients

BPD patients in BACCS were recruited from three sites, Toronto Canada, London UK and Dundee UK. BACCS was a community-based study, where subjects were recruited from psychiatric clinics, hospitals, primary care physicians and patient support groups. BPD patients were included in the study if they were over the age of 18 and had been diagnosed with Bipolar I or Bipolar II disorder as defined by the DSM-IV or ICD-10 (World Health Organisation, 1992; American Psychiatric Association, 2000). All patients were interviewed using the

Schedules for Clinical Assessment in Neuropsychiatry (SCAN) (Wing et al., 1998). All patients recruited in BACCS were euthymic (not in a clinically significant mood episode) at the time of interview and blood collection. All subjects were of White European parentage. Exclusion criteria include: first-degree relative having fulfilled criteria for schizophrenia; psychotic symptoms that were mood incongruent or present when there was no evidence for mood disturbance; intravenous drug use with a lifetime diagnosis of drug dependency; mania or depression occurring solely in relation to, or a consequence of, alcohol or substance abuse/dependence and/or medical illness; being related to an individual already included in the study. The BACC study was approved by ethics boards of participating centres and all patients provided written informed consent.

The current study utilized 40 BPD patient samples in total (30 in the discovery cohort and 10 in the replication cohort) collected only from the Dundee UK site, as this was the only site to collect blood for transcriptomic experiments. The subset used here was randomly selected from a larger group of samples. Further patient characteristics are detailed in Tables 3.1 and 3.2.

3.2.1.2 Major Depressive Disorder Patients

MDD patient samples were collected as part of the European study GENDEP, which is a 12-week partially randomized open label pharmacogenetic study. Patients were selected if they were diagnosed with MDD of at least moderate severity according to ICD-10 or DSM-IV criteria (World Health Organisation, 1992; American Psychiatric Association, 2000). Patients in GENDEP were aged

between 19–72 years and of White European parentage. Diagnoses were established using the semistructured SCAN interview (Wing et al., 1998). Exclusion criteria included personal and family history of schizophrenia or bipolar disorder; current substance dependence; being related to an individual already included in the study; known treatment resistance to both of the antidepressants given as part of the study. Patients with no contraindications were randomly allocated to flexible-dosage nortriptyline (50–150mg daily) or escitalopram (10–30 mg daily) for 12 weeks. Patients with contra-indications for one drug were offered the other. Blood was collected both at baseline and after eight weeks of treatment, which allows the study to draw comparisons from other clinical drug trials and pharmacogenetic studies using similar drug treatment durations. The GENDEP study was approved by ethics boards of participating centres, and all participants provided written informed consent.

The current study utilizes 46 patients in total (randomized to 30 in the discovery cohort and 16 in the replication cohort). The subset was selected and utilized for use in our previous studies (see Powell et al., 2012, 2013; see Chapter 4 and 6). Blood samples were collected both at the start of GENDEP and after eight weeks of treatment with escitalopram. All patients completed the Beck Depression Inventory at the time of blood collection (BDI; Beck & Steer, 1984). We utilised blood collected after eight weeks of treatment with escitalopram, for both our discovery and validation cohorts, and we did so for two reasons. Firstly, we have previously shown in this sample that escitalopram has no significant effect on the transcription of genes in the inflammatory cytokine pathway with the exception of ATP-binding cassette sub-family F member 1 (see Powell et al., 2013; Chapter 6), which will be excluded as a potential biomarker. Therefore,

medication is unlikely to act as a confounding factor in this sample. Secondly, we chose this time point, as it allowed us to adjust for the possible confounding effects of current mood state. Unlike the start of the GENDEP trial where all patient were in a clinically significant depressed state, after eight weeks of treatment, 26 patients still showed mild to moderate depression (defined here by BDI>10), whereas 20 patients were no longer in a clinically significant depressed state (defined here by BDI≤10). This allowed us to adjust our data for the effects of current mood state and draw comparisons with controls and BPD patients who were not in a clinically significant mood state at the time of blood collection. Further patient characteristics are shown in Tables 3.1 and 3.2.

We also utilised blood which was collected at the start of GENDEP to ascertain how stable transcriptional biomarkers were at differentiating MDD patients from other subject groups. At the start of GENDEP all patients had been drug-free for two weeks and were all in a clinically significant mood state (BDI>10). We assessed whether identified transcripts which were replicated in the validation cohort, continue to differentiate MDD patients from other subjects when blood is collected at a different time point, during different mood states, and during the absence of medication.

3.2.1.3 Control Subjects

Control subjects were selected from the Dundee, UK site, as part of BACCS. Control subjects were screened for lifetime absence of psychiatric disorder using a modified version of the Past History Schedule (McGuffin et al., 1986). All controls subjects were of white European parentage. Exclusion criteria were if

they; or a first-degree relative, ever fulfilled criteria for BPD, MDD or any other psychiatric disorder; if they had a BDI score of greater than 10 (Beck & Steer, 1984); did not return consent; failed to return cheek swabs or successfully give blood. The current study utilised 40 subject blood samples in total (30 in discovery cohort and 10 in replication cohort). Further subject characteristics are shown in Tables 3.1 and 3.2.

3.2.2 Experimental details

3.2.2.1 RNA Extraction

All blood samples from BACCS and GENDEP were collected in 10 ml PAXgene tubes (PreAnalytiX, Switzerland) and stored at -80°C. Prior to the start of gene expression studies, PAXgene tubes were allowed to thaw for 12 hours at room temperature. RNA extraction was performed using the Qiagen PAXgene Blood miRNA Kit (PreAnalytiX) following the standard manufacturer's protocol, see *Figure 3.1* for a summary of the extraction procedure. The purity and quantity of RNA was measured using the Nanodrop, ND1000 (Thermoscientific, Wilmington, DE). All samples had 260/280 ratios of between 1.9 and 2.3 [see Section 2.2.3.3 for more information]. RNA integrity numbers (RINs) were furthermore assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Berkshire, UK) and the average RIN was 8 ± 1.5 [see Section 2.2.3.4 for more information].

Subject Characteristic	BPD	MDD	Control	Total Sample
Sample number	30	30	30	90
Age (mean, (SD))	53.10 (14.17)	41.23 (12.53)	52.40 (14.35)	48.91 (14.62)
Males (n)	10	10	9	29
Females (n)	20	20	21	61
BMI (mean, (SD))	26.66 (5.51)	25.90 (4.11)	24.93 (3.33)	25.83
Cardiovascular Problem (n)	8	1	5	14
Diabetes (n)	2	0	2	4
Antidepressants (n)	6	30	0	36
Lithium (n)	20	0	0	20
Carbamazepine (n)	3	0	0	3
Sodium valproate (n)	3	0	0	3
Antipsychotics (n)	16	0	0	16

Table 3.1: A summary of subject characteristics in our discovery cohort. This includes general characteristics (total number in each subject group, age, number of males, number of females), information about co-morbidity (body mass index (BMI), number with diabetes, number with cardiovascular problems), and medication history (recent use of antidepressants, antipsychotics, lithium, carbamazepine, and sodium valproate).

Subject Characteristic	BPD	MDD	Control	Total Sample
Sample number	10	16	10	36
Age (mean, (SD))	52.50 (13.10)	45.19 (12.14)	54.6 (12.33)	49.83 (12.84)
Males (n)	2	7	3	12
Females (n)	8	9	7	24
BMI (mean, (SD))	24.68 (3.92)	26.01 (2.75)	28.33 (4.46)	26.22 (12.84)
Cardiovascular Problem (n)	3	4	0	10
Diabetes (n)	2	0	0	2
Antidepressants (n)	1	0	0	1
Lithium (n)	7	0	0	7
Carbamazepine (n)	2	0	0	2
Sodium valproate (n)	1	0	0	1
Antipsychotics (n)	3	0	0	3

Table 3.2: A summary of subject characteristics in our validation cohort. This includes general characteristics (total number in each subject group, age, number of males, number of females), information about co-morbidity (body mass index (BMI), number with diabetes, number with cardiovascular problems), and medication history (recent use of antidepressants, antipsychotics, lithium, carbamazepine, and sodium valproate).

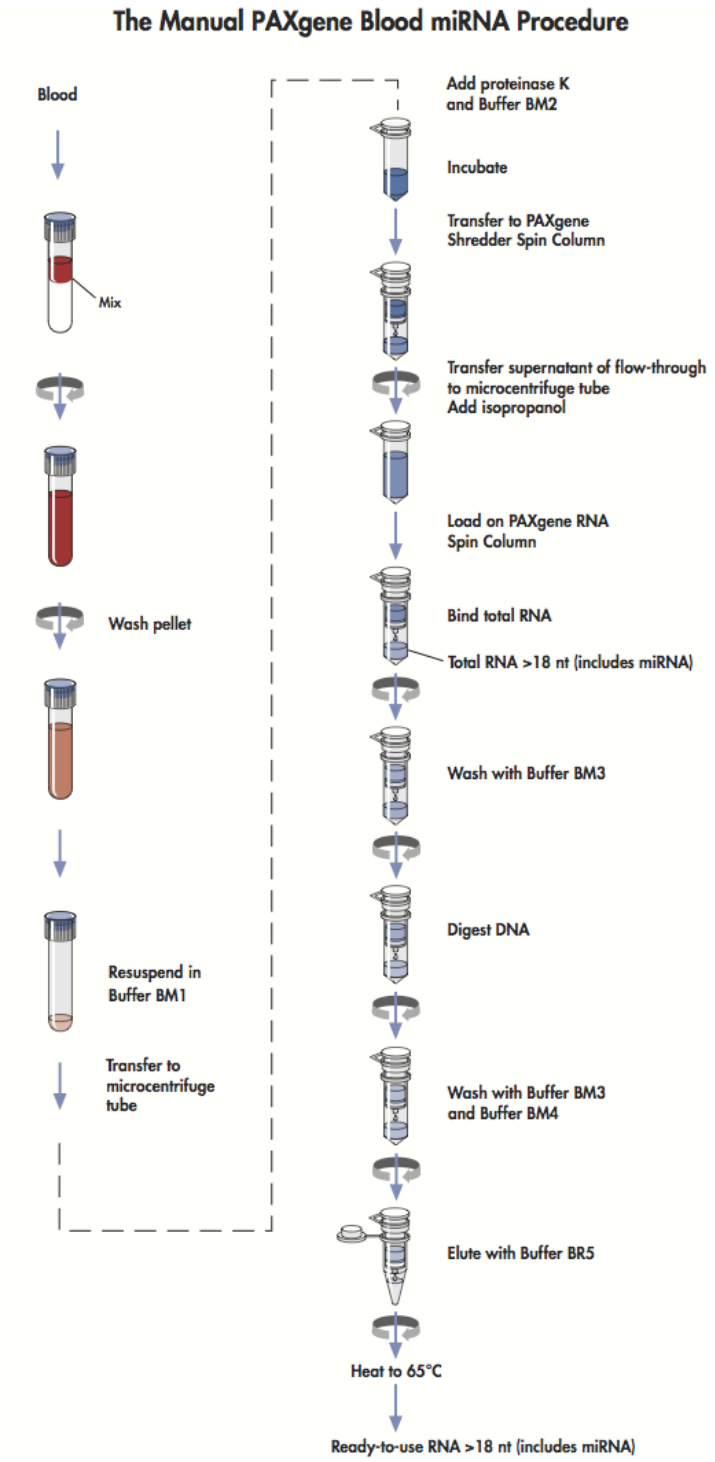


Figure 3.1: A summary of key steps involved in the extraction of RNA from whole blood stored in PAXgene tubes using the Qiagen PAXgene Blood miRNA Kit (PreAnalytiX).

3.2.2.2 Quantitative Polymerase Chain Reaction (qPCR)

A polymerase chain reaction (PCR) is a reaction in which a target sequence within a gene of interest is amplified through the actions of a DNA polymerase enzyme. qPCR involves the addition of a fluorescent dye which intercalates with double-stranded DNA allowing the amplification process to be detected in 'real time'. The 'cycle threshold' (C_t) value, is the key measurement in qPCR and refers to the number of amplification cycles required for a particular gene to reach a user-defined threshold of fluorescent signal, set above the level of background fluorescence.

The relative quantification method of normalization is often employed for qPCR data and requires the subtraction of relatively stable reference genes' expression from the potentially more dynamic expression of the target gene (Livak & Schmittgen, 2001). This process allows for comparisons across different samples, as the reference genes used for normalization are subject to the same conditions as the target gene itself; for example RNA integrity, reverse transcription efficiency and amount of starting material.

Reagents used in the qPCR component of the study were manufactured by SABiosciences (Frederick, MD, USA). Complementary DNA (cDNA) was prepared using 1 µg of total RNA and the SABiosciences RT^2 HT First Strand Kit following the manufacturer's protocol. Briefly, following genomic DNA removal, the samples were incubated for 15 minutes at 42°C with 6 µl of BC4 RT Mastermix (SABiosciences). The reverse transcriptase enzyme was subsequently inactivated at 95°C for 5 minutes. cDNA samples generated were stored at -20°C prior to use in the qPCR experiments.

Customized 384-well arrays were designed for qPCR experiments. These

arrays contained lyophilized primers for the 84 genes listed in the commercially available Human Inflammatory Cytokines & Receptors PCR Array (SABiosciences), with the addition of gene primers for interleukin-11 (*IL11)* and interleukin-6 (*IL6*) and the glucocorticoid receptor (*NR3C1*). Each array contained five housekeeping genes for normalization. These include: β2-microglobulin (*B2M*), Hypoxanthine phosphoribosyltransferase (*HPRT1*), Ribosomal protein L13a (*RPL13A*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*). Previous work has found that different mood disorder pharmacotherapies can differentially affect the expression of house keeping genes, impinging upon their usefulness as normalisation factors (see Powell et al., in press as provided in *Appendix B*). Thus, the three most stable housekeeping genes were selected based on RefFinder analyses and used for normalisation across samples.

Each 384-well array was designed to analyze four samples simultaneously. The qPCR reagents used consisted of: 550 µl of 2X SABiosciences RT^2 qPCR Master Mix (SYBR green), 102 µl of diluted synthesized cDNA and 448 µl RNAse free water, with a total volume of 1100 µl for each sample. 10 µl was then added to each well on the array. Each qPCR array contained the following controls: human genomic DNA control (gDNA), reverse transcription control (RTC) and a positive PCR control (PPC). To ascertain whether samples passed quality control checks for gDNA and RTC, the manufacturer's quality control criteria were applied. The qPCR reactions were performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, California, USA). Thermal cycling conditions consisted of an enzyme activation stage (95 °C for 10 minutes), followed by 40 cycles of a denaturation stage (95°C for 15 secs) and a

hybridization and extension stage (65°C for 1 minute). The software program SDS 2.3 (Applied Biosystems) generated cycle threshold values (C_t) from the data collected

3.3 Statistical Analysis

Generated C_t values of greater than 37 were removed and excluded from further analysis as such high C_t values are indicative of very low expression levels. Furthermore, if as a result of data removal, a gene showed missing data for more than half of the total patient sample, that gene was excluded from further analysis. The relative expression of target genes was calculated by subtracting the mean C_t of the selected reference genes from the C_t of the target gene to generate ΔC_t values (Livak & Schmittgen, 2001). The three most stable housekeeping genes were selected as reference genes for normalization purposes based on RefFinder analyses, which is available as a web-based tool (http://www.leonxie.com/referencegene.php), see *Appendix B* for more details. Relative expression values were then adjusted for PPC (to account for any inter-plate variability), age, sex, current mood status, BMI and the presence of comorbid disorders (diabetes, cardiovascular problems). ΔC_t were used in statistical calculations, and $2^{-\Delta C_t}$ were used to generate bar charts (Livak & Schmittgen, 2001).

To ascertain whether significant transcriptional differences existed between control, MDD and BPD subject groups in our discovery cohort, we performed analysis of variance (ANOVA) tests. Partial eta squared (η_p^2) was calculated as an estimate of effect size, by dividing the sum of squares between

groups by the total sum of squares. Games-Howell post-hoc tests were subsequently performed to correct for multiple testing and to generate pair-wise comparisons between subject groups (Games & Howell, 1976). Based on results from the discovery cohort, we then performed one-tailed independent sample t-tests to attempt to replicate findings in our validation cohort.

We have previously shown that escitalopram does not affect the transcription of inflammatory cytokines in our MDD patient sample, with the exception of ATP-binding cassette sub-family F member 1, which has been excluded as a potential biomarker (see Powell et al., 2013, or Chapter 6). However, medications used in our BPD patient sample may affect transcription, and as such we performed post-hoc analyses to assess whether these medications may represent confounding factors. Consequently, for each gene's expression that significantly differentiated our BPD subjects from either controls or MDD patients, we ran univariate linear regressions for our BPD sample only. The expression of the significant gene was selected as the dependent variable and regularly used medications included as covariates. For any medications which significantly predicted the expression of a gene, we excluded that transcript as a likely biomarker.

For any transcripts that significantly differentiated MDD patients from control subjects or BPD patients, in both the discovery and validation cohorts, we performed an additional test to determine the stability of these transcripts as state biomarkers for MDD. We achieved this by utilising transcript data generated from blood collected at a different time point (start of GENDEP), under different conditions (patients were drug free, all patients were in a depressed episode). As before, we attempted to validate biomarkers by

performing one-tail independent samples t-tests.

3.4 Results

3.4.1 Validation of internal controls

All qPCR plates passed quality control checks outlined. 70 out of 87 target genes were sufficiently detectable according to our criteria. RefFinder analyses revealed that *B2M*, *RPL13A* and *ACTB* were the three most stable housekeeping genes across all samples and subsequently were selected for normalization purposes, see Figure 3.2.

3.4.2 Transcriptional differences between subject groups

3.4.2.1 Discovery Cohort

ANOVA revealed 11 genes which showed nominally significant transcriptional differences ($p \leq 0.05$) between our three subject groups. The most significant differences between subject groups were found in Chemokine (C-C motif) ligand 24 [*CCL24:* $F(2, 85) = 6.438$, $p = 0.002$, $\eta_p^2 = 0.134$] and interleukin-8 [*IL8:* $F(2, 87) = 6.872$, $p = 0.002$, $\eta_p^2 = 0.136$], see *Appendix C* for full ANOVA results. Games-Howell post hoc analyses were subsequently performed on all genes present on the array, see *Appendix D* for full results. These tests correct for the effects of multiple testing, and generate pairwise comparisons. Table 3.3 lists the

genes which produced significant p-values from the ANOVA analyses ($p \leq 0.05$) and details corrected pair-wise results generated from Games-Howell post hoc analyses. None of the medications used by our BPD patients significantly affected the transcription of any of the potential biomarkers identified in our discovery cohort.

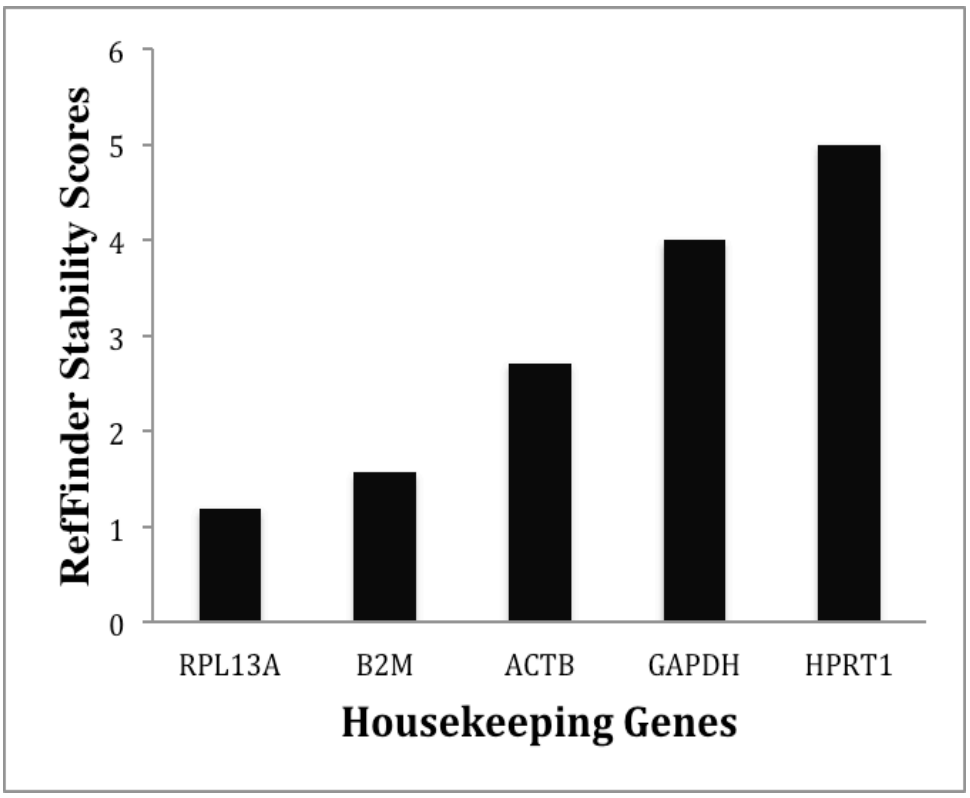


Figure 3.2: A bar chart showing the results of RefFinder analyses performed on a panel of five housekeeping genes. The gene names of the housekeeping genes are indicated on the x-axis, along with their expression stability score shown on the y-axis. Lower RefFinder stability scores represent more stable reference genes.

Discovery Cohort															
Gene	MDD v Control					MDD v BPD					BPD v Controls				
	$\bar{x}$ difference	S.E.	p-value	95% C.I.		$\bar{x}$ difference	S.E.	p-value	95% C.I.		$\bar{x}$ difference	S.E.	p-value	95% C.I.	
CCL24	-0.779	0.259	**0.011**	-1.404	-0.153	-0.676	0.249	**0.025**	-1.280	-0.072	-0.102	0.181	0.839	-0.538	0.334
CCR4	0.615	0.255	**0.049**	0.001	1.229	0.507	0.278	0.170	-0.161	1.175	0.108	0.236	0.891	-0.460	0.676
CCR6	0.510	0.192	**0.028**	0.047	0.973	0.091	0.215	0.907	-0.426	0.607	0.419	0.205	0.111	-0.074	0.913
CCR9	0.644	0.249	**0.032**	0.046	1.242	0.276	0.247	0.507	-0.318	0.870	0.368	0.256	0.327	-0.247	0.983
CXCL1	0.738	0.261	**0.017**	0.111	1.366	0.353	0.276	0.413	-0.311	1.017	0.385	0.274	0.345	-0.274	1.045
CXCL6	1.007	0.347	**0.015**	0.167	1.848	0.549	0.377	0.319	-0.358	1.456	0.459	0.294	0.270	-0.248	1.166
CXCL9	0.517	0.347	0.303	-0.318	1.352	1.004	0.317	**0.007**	0.239	1.770	-0.487	0.274	0.187	-1.149	0.174
CXCL10	1.094	0.372	**0.013**	0.197	1.991	0.944	0.421	0.072	-0.068	1.956	0.150	0.388	0.921	-0.784	1.084
XCR1	-0.109	0.226	0.881	-0.656	0.438	-0.772	0.262	**0.013**	-1.404	-0.141	0.664	0.214	**0.009**	0.148	1.180
IL8	1.021	0.303	**0.004**	0.292	1.749	0.026	0.323	0.997	-0.750	0.802	0.995	0.316	**0.007**	0.234	1.756
NR3C1	0.543	0.202	**0.025**	0.057	1.028	0.170	0.208	0.696	-0.332	0.672	0.373	0.222	0.221	-0.161	0.906

Table 3.3: A table detailing Games-Howell pair-wise post-hoc analysis results for genes which produced significant p-values ($p \leq 0.05$) in ANOVAs from our discovery cohort. The table details results from pairwise comparisons between subject groups, including the mean differences in relative expression between subject groups, the standard error (S.E.), p-value, and 95% confidence interval (95% C.I.). Significant pairwise comparisons ($p \leq 0.05$) are highlighted in bold.

3.4.2.2 Validation Cohort

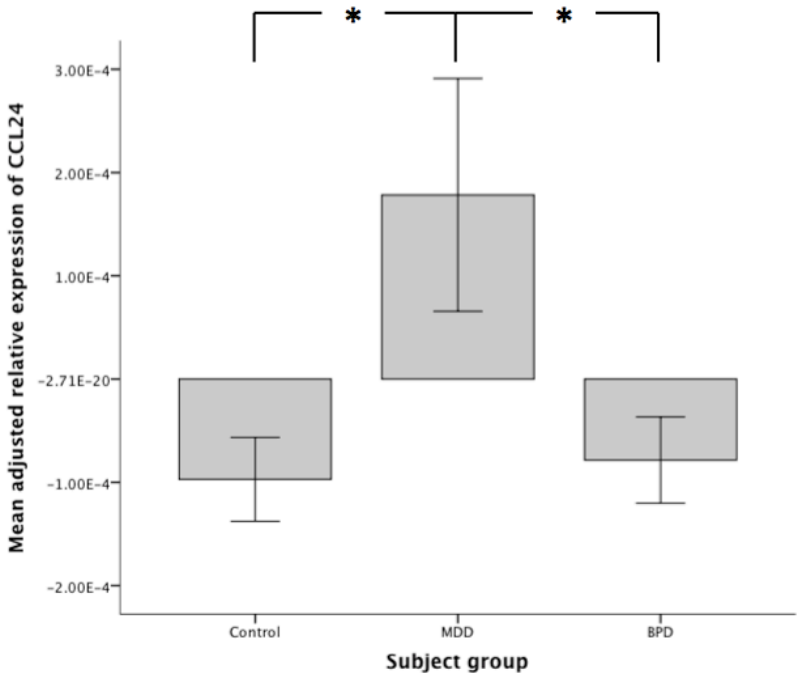
One-tailed independent t-tests were used in our validation cohort to test whether we could replicate potential biomarkers identified from our discovery cohort, see Table 3.4. Higher transcription of *CCL24,* the most significant difference revealed between subjects in our discovery cohort, again significantly differentiated MDD patients from controls ($t = 2.394$, d.f. = 23, $p = 0.0125$) and BPD patients ($t = 2.674$, d.f. = 23, $p = 0.007$) in the validation cohort, see Figure 3.3. Lowered transcription of *CCR6* also continued to differentiate MDD patients from controls in our validation cohort ($t = -2.315$, d.f. = 23, $p = 0.015$), see Figure 3.4.

We additionally used one-tailed independent samples t-tests to observe whether *CCL24* and *CCR6* transcription continued to differentiate MDD patients from our other subject groups when blood was collected from a different time point. Again, we found that higher transcription of *CCL24* significantly differentiated MDD patients from controls in our discovery cohort ($t = 7.237$, d.f. = 57, $p \leq 0.000001$) and replication cohort ($t = 6.603$, d.f. = 23, $p \leq 0.000001$) when MDD blood was collected from different time point. Similarly, we found that higher transcription of *CCL24* significantly differentiated MDD patients from BPD patients in both our discovery cohort ($t = 7.247$, d.f. = 57, $p \leq 0.000001$) and replication cohort ($t = 4.511$, d.f. = 11.64, $p \leq 0.001$). Additionally, lower transcription of *CCR6* continued to differentiate MDD patients from controls in both our discovery cohort ($t = -1.841$, d.f. = 58, $p = 0.035$) and replication cohort ($t = -1.799$, d.f. = 23, $p = 0.043$).

Validation Cohort										
Gene	MDD v Control			MDD v BPD			BPD v Control			Replication?
	t	df	p	t	df	p	t	df	p	
CCL24	2.394	23	**0.013**	2.674	23	**0.007**	-	-	-	Y
CCR4	-1.218	23	0.118	-	-	-	-	-	-	N
CCR6	-2.315	23	**0.015**	-	-	-	-	-	-	Y
CCR9	1.073	23	0.147	-	-	-	-	-	-	N
CXCL1	-0.455	23	0.327	-	-	-	-	-	-	N
CXCL6	-1.542	22	0.079	-	-	-	-	-	-	N
CXCL9	0.066	23	0.474	-	-	-	-	-	-	N
CXCL10				0.214	22	0.416	-	-	-	N
XCR1	-0.998	23	0.165	-	-	-	-0.879	18	0.391	N
IL8	-	-	-	1.331	23	0.098	-0.347	18	0.367	N
NR3C1	-0.359	23	0.362	-	-	-	-	-	-	N

Table 3.4: Table detailing result from the one-tailed t-tests performed on our validation cohort, including t-values, degrees of freedom (d.f.), and p-values. The transcripts which significantly differentiated between subjects groups, replicating results from our discovery cohort, are highlighted in bold and indicated with a Y under 'Replication?'.

A.



B.

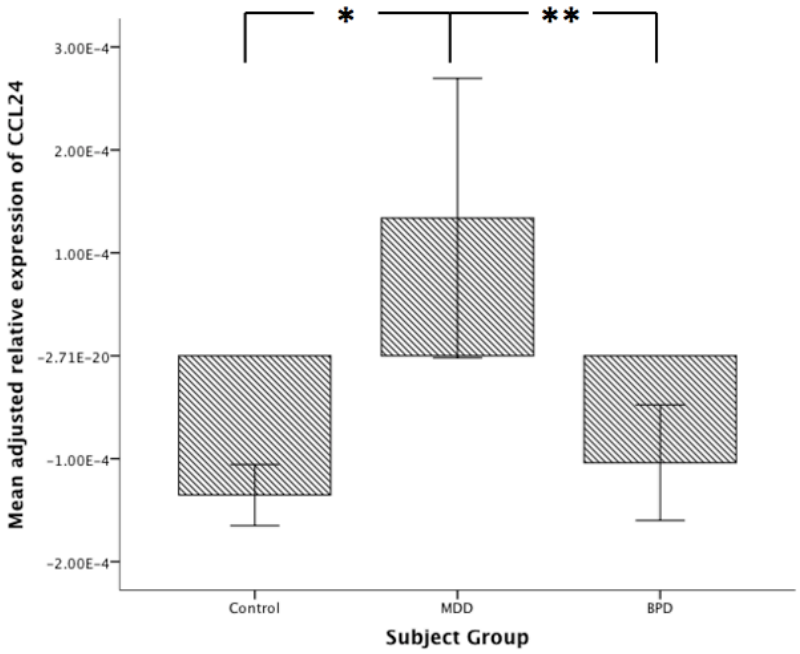
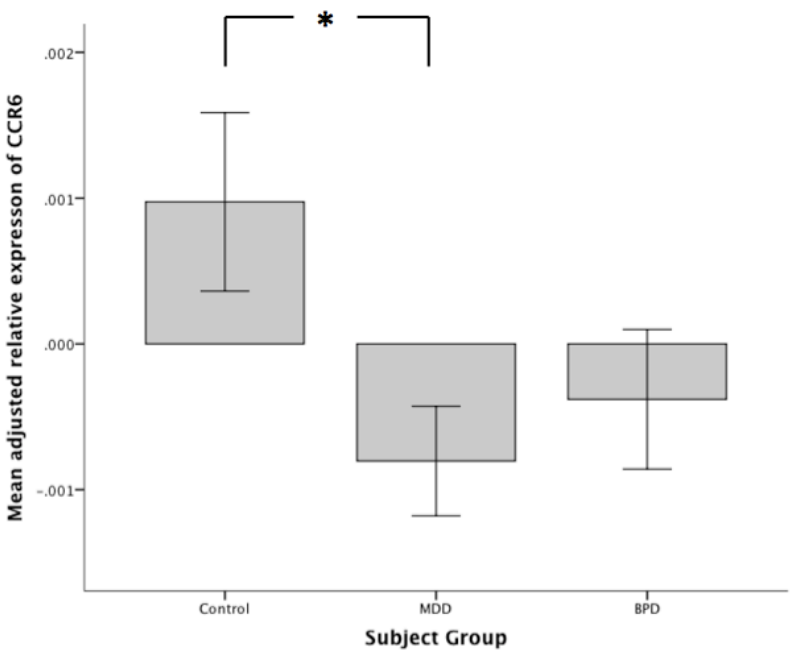


Figure 3.3: Bar charts showing the adjusted mean relative expression of *CCL24* (y-axis) in our control subjects, MDD patients and BPD patients (x-axis) using data collected from our (A) Discovery cohort, and (B) Validation cohort. Error bars represent standard error. Significant differences between subject groups are indicated with a *.

A.



B.

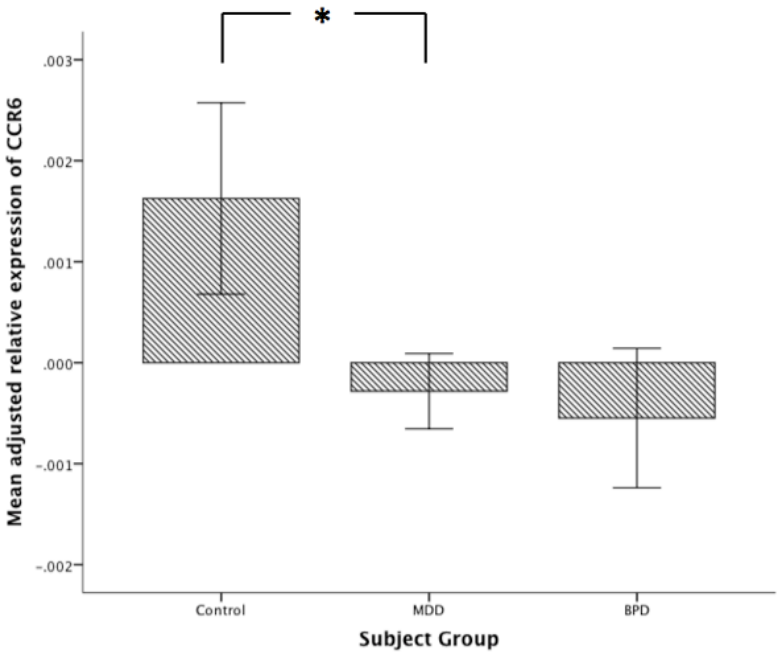


Figure 3.4: Bar charts showing the adjusted mean relative expression of *CCR6* (y-axis) in our control subjects, MDD patients and BPD patients (x-axis) using data collected from our (A) Discovery cohort, and (B) Validation cohort. Error bars represent standard error. Significant differences between subject groups are indicated with a *.

3.5 Discussion

Mood disorders are heterogeneous disorders that are diagnosed through patients displaying a number of clinical characteristics. The absence of a specific and objective diagnostic test has led to relatively high rates of misdiagnosis for mood disorders, particularly between MDD and BPD patients (Lewis et al., 2003). Recent reports have revealed differences in cytokine gene expression between MDD patients and controls, and BPD patients and controls (Padmos et al., 2008; Cattaneo et al., 2012). This follows a growing body of evidence linking inflammation with mood disorder pathophysiology and response to mood disorder pharmacotherapies (Raison et al., 2006; Dantzer et al. 2008; Uher et al., 2010; Powell et al., 2012b; Powell et al., 2013a, Powell et al., 2013b).

Here, we attempted to identify transcriptional differences in the inflammatory cytokine pathway between MDD, BPD and control subjects in a 'discovery cohort', and to assess whether these differences might act as biomarkers to differentiate between subject groups in a 'validation cohort'. Results from our discovery cohort revealed 11 transcripts which differentiated between our subject groups (see *Table 3.3*). The majority of these transcripts coded for chemokines and chemokine receptors. However, two notable exceptions include interleukin-8 (*IL8*) and the glucocorticoid receptor (*NRC31*). Previous reports have found lowered levels of IL-8 protein in the blood of MDD patients relative to controls, and within the cerebrospinal fluid of suicide attempters compared to controls (Simon et al., 2008; Lehto et al., 2010; Isung et al., 2012). In the current study we found that lower transcription of *IL8* distinguished both types of mood disorder patient (MDD and BPD) from control subjects (see *Table 3.3*). This may suggest that a common molecular

pathway impacting upon the transcription of *IL8* could be involved in mood disorder pathophysiology. We also found that MDD patients exhibited decreased transcription of *NRC31* relative to control subjects (see *Table 3.3*). Lowered expression of NRC31 has previously been reported both at the protein and transcriptional level amongst MDD patients, and altered expression and functionality of NRC31 has a recognised role in the pathophysiology of MDD (Pariante & Miller, 2001). However, neither *IL8* nor *NRC31* transcripts significantly differentiated between subject groups in our validation cohort, which suggests that although they may be involved in mood disorder pathophysiology, they may not be reliable or specific enough to be utilised as biomarkers.

In contrast, higher transcription of *CCL24* consistently differentiated MDD patients from control and BPD subjects, and lower transcription of *CCR6* consistently differentiated MDD patients from controls, in both our discovery and replication cohorts (see *Figure 3.3* and *Figure 3.4*). The transcription of these genes continued to differentiate MDD patients from other subject groups even when MDD blood was utilised from a different time point (see *Section 3.4 Results*), corroborating the notion that transcriptional differences in these genes likely relate to long-lasting state differences associated with MDD, as opposed to trait differences.

Both *CCL24* and *CCR6* code for genes in the chemokine cytokine family. The chemokines are small 'chemotactic' cytokines that facilitate the migration of immune cells (e.g. to a site of infection; Lehto et al., 2010). *CCL24* codes for a chemokine which is chemotactic for resting T lymphocytes, eosinophils, and to a lesser extent neutrophils (Patel et al., 1997; White et al., 1997). In contrast, *CCR6* codes for a G-protein coupled receptor present on

immature dendritic cells, B-cells and memory t-cells, and binds macrophage inflammatory protein 3 alpha (Ai et al., 2004). Chemokines have previously been implicated as potentially important cytokines in the pathophysiology of MDD and higher levels of chemokine proteins have previously been revealed amongst MDD patients relative to controls (Miller et al., 2008; Lehto et al., 2010). However, this is the first study to identify *CCL24* and *CCR6* transcripts as potential diagnostic biomarkers.

In addition to gene transcription offering a more objective method of clinical diagnosis, the fact that it is also a continuous measure gives it certain advantages over currently utilised categorical measures. For instance, continuous or dimensional diagnostic measures are believed to be more stable over time, offer a better measure of symptom severity, and be better predictors of comorbidity and chronicity (Clark et al., 1995; Watson, 2005). Consequently, transcriptional measures, such as those reported here, could be combined with phenomenological or symptom dimension measures in future diagnostic manuals to more sensitively capture clinically useful information for MDD and BPD diagnosis.

Although results reported here are promising, there are four main limitations to this study. Firstly, this study utilises relatively small sample sizes, and although we use both a discovery and replication cohort, patients were obtained as subsamples from the same studies, so it only offers a pseudo-independent replication. Therefore replication studies are required in a larger independent sample. Secondly, although we considered the effects of different medications on gene expression profiles, all of our patients were medicated. Based on our previous work on the MDD patient sample used here, we can, with

some confidence, rule out the confounding effects of escitalopram treatment (see Powell et al., 2013). This was further supported by analyses on our MDD patients after they were medication-free for two weeks (see *Section 3.4 Results*). However, our BPD patient cohort were all treated with a variety of medications, and although we could rule out the confounding effects of each medication separately, we could not assess whether common actions of different medications may have confounding effects on gene transcription in our sample. Therefore future studies in drug-free patients are required in order to validate the transcript biomarkers identified in this study. Thirdly, although we accounted for differences in BMI, cardiovascular problems and diabetes between our subject groups, we did not have an extensive account of comorbidities. Comorbid ailments such as chronic pain, irritable bowel syndrome and arthritis are known to be more frequent amongst mood disorder patients and they likely affect cytokine expression (Leboyer et al., 2012), and thus a more extensive list of comorbid disorders should be accounted for in future studies. Finally, without cell count information we cannot determine the cell types that may be driving our observed transcript differences between subjects.

Despite its limitations, the current study utilises well-characterized clinical samples, stringent quality control steps, normalisation protocols and statistical analyses. This study supports previous reports of differences in the expression of *IL8* and *NR3C1* amongst mood disorder patients. However, the lack of replication in our validation cohort suggests that differences in the transcription of these genes may not be reliable enough to be utilised as biomarkers. Instead, this study emphasized the potential importance of chemokines as biomarkers, and specifically it identifies the potential utility of *CCL24* and *CCR6* transcripts

as novel biomarkers differentiating MDD patients from control subjects and BPD patients. If replicated in other studies, these transcripts could be used in conjunction with symptom measures to more accurately diagnose MDD from the outset and differentiate MDD patients from non-depressed subjects and BPD patients.

Chapter 4

Identifying transcriptomic biomarkers in the inflammatory cytokine pathway for clinical response to the antidepressant escitalopram

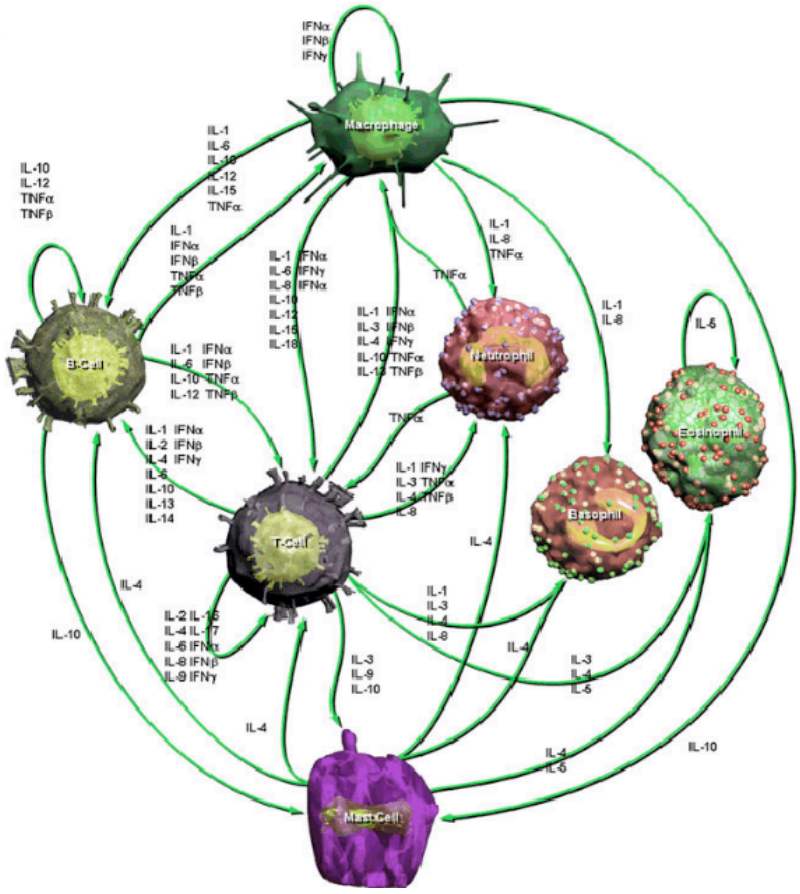


Figure 4.0: A picture showing the complex interactions between immune cells, orchestrated through the release of different types of inflammatory cytokines. Adapted from http://www.sabiosciences.com/pathway.php?sn=Cytokine_Network .

4.0 Notes

This chapter consists of a manuscript published in *European Neuropsychopharmacology* in November 2012. For more information on the pressing need for biomarkers as predictors of antidepressant response, see *Sections 1.4.2.* For more information on previous work relating to cytokines as biomarkers for antidepressant response see *Section 1.5.1.2.* For more information on RNA see *Section 2.3.3.1.* For more information on RNA quality controls steps see *Section 2.2.3.3.* and *2.2.3.4.* For more information on qPCR methodology, see *Section 3.2.2.2.*

For consistency with the wider thesis, *Section 1. Introduction* in this manuscript is referred to as Section 4.1 elsewhere, *Figure 1* within the manuscript will be referred to as Figure 4.1, *Table 1* will be referred to as Table 4.1 etc. geNorm and Normfinder analyses described in this manuscript are an earlier variation of the RefFinder analyses described in Section 3.3, used for reference gene selection. The Supplementary Information mentioned in the manuscript is provided in *Appendix E.*

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Tumor necrosis factor and its targets in the inflammatory cytokine pathway are identified as putative transcriptomic biomarkers for escitalopram response

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KEYWORDS
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Biomarker

Abstract
Converging evidence suggests that the activation of the inflammatory cytokine pathway is important in the pathophysiology of unipolar depression. Antidepressants have anti-inflammatory properties and evidence suggests that inter-individual variability in response to antidepressants may reflect genetic differences in the inflammatory cytokine pathway. In particular, protein levels of Tumor Necrosis Factor (TNF) and the SNPs rs1126757 in interleukin-11 (*IL11*), and rs7801617 in interleukin-6 (*IL6*), have previously been implicated in the clinical response to the selective serotonin reuptake inhibitor (SSRI) antidepressant escitalopram. This study investigated the transcription of *TNF*, *IL11* and *IL6* as well as genes in the wider inflammatory cytokine pathway both at baseline and after escitalopram treatment in depressed patients who were either clinical "responders" (n=25) or "non-responders" (n=21). Samples were obtained as a subset of the Genome-Based Therapeutic Drugs for Depression (GENDEP) project and response status is based on changes in the Montgomery–Asberg Depression Rating Scores over a 12 wk treatment period. Binary logistic regressions revealed significant expression differences at baseline between responders and non-responders in *TNF*, and after escitalopram treatment in *TNF* and *IL11*. Differences in *IL11* after treatment were found to be driven by drug-induced allele-specific expression differences relating to rs1126757. Top hits in the wider inflammatory cytokine pathway at both baseline and after escitalopram treatment were found to be targets of TNF. The current study adds substantial support for the role of the inflammatory

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cytokine pathway in mediating response to the SSRI escitalopram, and is the first to identify *TNF* and its targets as putative transcriptomic predictors of clinical response.

1. Introduction

Converging evidence suggests that the activation of the inflammatory cytokine pathway is important in the pathophysiology of unipolar depression. Research has shown that an increase in proinflammatory cytokines, acute-phase proteins and chemokines are present in individuals suffering from depression (Miller et al., 2008). Interestingly, antidepressants have anti-inflammatory properties (Abdel-Salam et al., 2003; Roumestan et al., 2007) and evidence suggests that the inter-individual variability in response to antidepressants may reflect genetic differences in the inflammatory cytokine pathway (Uher et al., 2010).

Three important candidates in the inflammatory cytokine pathway have been linked to the clinical response to the selective serotonin reuptake inhibitor (SSRI) escitalopram; these include interleukin-6 (IL6), interleukin-11 (IL11) and tumor necrosis factor (TNF/TNF-α). Serum protein levels of both IL6 and TNF have shown to be increased in individuals with depression compared to controls (Dowlati et al., 2010; Kahl et al., 2002). Furthermore, serum levels of both of these inflammatory cytokines have been shown to be highest in patients resistant to antidepressant treatment (Maes et al., 1997; Eller et al., 2008). Recent evidence also suggests that differences between responders and non-responders to antidepressants may be genetically mediated. For instance, a candidate gene association study as part of the human component of the pharmacogenetic Genome-Based Therapeutic Drugs for Depression (GENDEP) project revealed a single nucleotide polymorphism (SNP) in *IL6* (rs7801617) to predict escitalopram response (Uher et al., 2010). Similarly, as part of the genome-wide association study (GWAS), a SNP in *IL11* (rs1126757) was found to predict clinical response to escitalopram (Uher et al., 2010).

As opposed to genotype biomarkers, transcriptional biomarkers have the advantage of capturing the functional output of genotypes interacting with epigenetic and transcription factor binding; both of which have additionally been linked to antidepressant response (Cassel et al., 2006; Thome et al., 2000). As such, data collected from transcriptomic studies lie closer to the phenotype in question and may better represent the cause of differential responses to drugs. By considering the transcription of genes in blood at baseline (i.e. prior to treatment with escitalopram), it could lead to the identification of peripherally accessible response biomarkers which could translate clinically to patient-specific treatment selection. Furthermore, considering transcriptional differences between responders and non-responders after treatment with escitalopram could reveal the drug-induced transcriptional differences and pathway differences between these two groups.

The current study aimed to investigate transcriptomic differences between responders and non-responders to escitalopram using a subset of GENDEP samples. Expression differences between responders and non-responders in the genes *IL11*, *IL6* and *TNF* were considered as well as genes in the broader inflammatory cytokine pathway both before and after treatment with escitalopram. As previous pharmacogenetic studies undertaken as part of GENDEP have implicated rs7801617 in *IL6* and rs1126757 in *IL11* as affecting clinical response to escitalopram (Uher et al., 2010), any significant expression differences in *IL6* or *IL11* were further considered in relation to genotype at these SNPs.

2. Experimental procedures

2.1. Clinical sample

Patient samples were taken from the Genome-Based Therapeutic Drugs for Depression (GENDEP) project which has been described in detail elsewhere (Uher et al., 2009). Briefly, GENDEP is a 12-wk partially randomized open label pharmacogenetic study with two active treatment arms. A total of 868 treatment seeking adults (men: n=321; women: n=547) with unipolar depression of at least moderate severity according to ICD-10 or DSM-IV criteria were recruited from nine European centers. Patients were aged 19–72 years and of Caucasian European parentage. Diagnoses were established using the semistructured Schedules for Clinical Assessment in Neuropsychiatry interview (Wing et al., 1998). Exclusion criteria were personal and family history of schizophrenia or bipolar disorder, current substance dependence, or if participants had previously taken both of the drugs and previously demonstrated treatment resistance. Eligible participants were allocated to treatment with either the selective serotonin reuptake inhibitor escitalopram (total n=394) or the norepinephrine reuptake inhibitor nortriptyline (total n=312) that differ by antidepressant mechanisms of action. Patients with no contraindications were randomly allocated to flexible-dosage nortriptyline (50–150 mg daily) or escitalopram (10–30 mg daily) for 12 weeks. Patients with contraindications for one drug were offered the other. Blood was collected both at baseline and after eight weeks of treatment, which allows the study to draw comparisons from other clinical drug trials and pharmacogenetic studies using similar drug treatment durations. This study focuses on the effects of the selective serotonin reuptake inhibitor (SSRI) escitalopram only. This was chosen because the genome wide association study previously performed on GENDEP (Uher et al., 2010) revealed polymorphisms within inflammatory cytokines (the genes focused on in this study) predicted response to escitalopram but not nortriptyline. This subsequently provided the rationale for investigating the role of transcriptional biomarkers in the inflammatory cytokine pathway for escitalopram response. The GENDEP project was approved by ethics boards of participating centers, and all participants provided written informed consent.

Participants were assessed for severity of depressive symptoms using the clinician-rated Montgomery-Åsberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979) on a weekly basis. Previous work by Uher et al. (2008) found that MADRS scores were the most sensitive, clinically representative and internally consistent scores for depression symptom changes in GENDEP, and consequently is the measure we utilize in this study. Percentage changes in MADRS scores from baseline to week 12 were used to dichotomize patients into "responders" or "non-responders". For the purposes of this study, those patients with a percentage MADRS change of greater than 50% were considered responders, whereas those with a percentage

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MADRS change of less than 50% were considered non-responders. A subset of 46 patients from GENDEP were selected based on four criteria: (a) patients were treated with escitalopram, (b) patients had complete clinical data for the full 12 wk treatment period, (c) blood was collected from these patients both at baseline and at week 8, (d) the subset contained approximately equal numbers of responders (n=25) and non-responders (n=21).

The sample consists of n=17 males and n=29 females. The average age of participants at the start of the study was 42.6±12.4 (years, S.D.). The average baseline severity of depression in the sample was 29.8±5.3 (MADRS score, S.D.). All participants were drug-free for at least two weeks prior to the start of the study. Prior to those two weeks participants had been taking a variety of medications including: antidepressants (n=12), benzodiazepines (n=21) and hypnotics (n=5).

2.2. RNA extraction

Ten milliliter of blood was collected in PAXgene tubes (PreAnalytiX, Switzerland) at baseline and again after eight weeks of treatment with escitalopram and frozen at −80 °C. Prior to the start of gene expression studies, PAXgene tubes were allowed to thaw for 12 h at room temperature. RNA extraction was performed using the Qiagen PAXgene Blood miRNA Kit (PreAnalytiX) following the standard manufacturer's protocol. The purity and quantity of RNA was measured using the Nanodrop, ND1000 (Thermoscientific, Wilmington, DE). All samples had 260/280 ratios of between 1.9 and 2.3. RNA integrity numbers (RINs) were furthermore assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Berkshire, UK) and the average RIN was 8±1.5.

2.3. Quantitative PCR

Reagents used in the quantitative PCR (qPCR) component of the study were manufactured by SABiosciences (Frederick, MD, USA). Copy DNA (cDNA) was prepared using 1 μg of total RNA and the SABiosciences RT^2 HT First Strand Kit following the manufacturer's protocol. Briefly, following genomic DNA removal, the samples were incubated for 15 min at 42 °C with 6 μl of BC4 RT Mastermix (SABiosciences). The reverse transcriptase enzyme was subsequently inactivated at 95 °C for 5 min. cDNA samples generated were stored at −20 °C prior to use in the qPCR experiments.

Customized 384-well arrays were designed for qPCR experiments. These arrays contained lyophilized primers for the 84 genes listed in the commercially available Human Inflammatory Cytokines & Receptors PCR Array (SABiosciences), with the addition of gene primers for *IL11* and *IL6*. Each array contained five housekeeping genes for normalization which included: β2-microglobulin (*B2M*), Hypoxanthine phosphoriboseyltransferse (*HPRT1*), Ribosomal protein L13a (*RPL13A*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*). As previous studies have shown that treatment with escitalopram can affect the stability of housekeeping gene expression (Sugden et al., 2010), geNorm and NormFinder analyses were used to select the most appropriate reference genes from the five housekeeping genes included in the arrays (Vandesompele et al., 2002; Andersen et al., 2004).

Each 384-well array was designed to analyze four samples simultaneously. As such (where possible), each array contained cDNA from one responder and one non-responder, both at baseline and after eight weeks of escitalopram treatment. The qPCR reagents used consisted of: 550 μl of 2× SABiosciences RT2 qPCR Master Mix (SYBR green), 102 μl of diluted synthesized cDNA and 448 μl RNAse free water, with a total volume of 1100 μl for each sample.

Each qPCR array contained the following controls: human genomic DNA control (gDNA), reverse transcription control (RTC) and a positive PCR control (PPC). To ascertain whether samples passed quality control checks for gDNA and RTC, the manufacturer's quality control criteria were applied. To establish whether reverse transcription was successful for each sample the following criterion had to be met: (Average RTC C_t−Average PPC C_t)≤5. To ensure all samples were free from gDNA contamination, the gDNA control must produce a C_t≥35.

The qPCR reactions were performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, California, USA). Thermal cycling conditions consisted of an enzyme activation stage (95 °C for 10 min), followed by 40 cycles of a denaturation stage (95 °C for 15 s) and a hybridization and extension stage (65 °C for 1 min). The software program SDS 2.3 (Applied Biosystems) generated cycle threshold values (C_t) from the data collected.

2.4. Genotype data

Genotype data was collected as part of the genome-wide association study (Uher et al., 2010). Full details of genotyping and quality controls measure can be found elsewhere (Uher et al., 2009). Briefly, samples were sent to the Centre National de Genotypage (Evry Cedex, France) and genotyped using the Illumina Human610-Quad bead chip (Illumina, Inc., San Diego, CA, USA). This chip assays more than 610,000 SNPs selected to provide a comprehensive coverage across populations. All 46 individuals passed routine quality controls which included removing individuals for ambiguous sex, abnormal heterozygosity, cryptic relatedness (up to third degree relatives), genotyping incompleteness (less than 97% coverage) and non-white European admixture. Genotype data includes the top SNP from the GWAS, rs1126757. Genotype data for this SNP was subsequently extracted using gPLINK (Purcell et al., 2007).

2.5. Statistical analysis

C_t values of greater than 37 were removed and excluded from further analysis as such high C_t values are indicative of very low expression levels. Furthermore, if a gene showed barely detectable levels of expression and produced missing data for more than $^1/_3$ of the total patient sample, that gene was excluded from further analysis. The relative expression of target genes was derived using the formula $2^{-\Delta C_t}$, where delta C_t (ΔC_t) is the difference between the C_t of the target gene and the mean C_t of the selected reference genes (Livak and Schmittgen, 2001). The differences in the relative expression of each gene between responders and non-responders were determined using binary logistic regressions, covarying for age, sex, center of treatment and baseline MADRS score in SPSS Version 15 (SPSS Inc., Chicago, IL).

A univariate linear regression was used to test if rs1126757 genotype was affecting *IL11* expression after covarying for age, sex and center of recruitment. Other potential confounding factors, such as smoking, body mass index, medication history and anxiety symptoms, were found not to significantly affect relative expression of TNF, IL11 or IL6 and were therefore not included in the model as covariates. False Discovery Rate (FDR) was used to establish if the relative expression of any genes in the hypothesis-free component showed significant differences between responders and non-responders. Ingenuity Pathway Analysis (Ingenuity Systems, Redwood, CA) was performed on all genes that produced uncorrected p-values of p≤0.05 at baseline or week 8 when comparing responders and non-responders.

3. Results

3.1. Validation of internal controls

All qPCR plates passed quality control checks and all positive PCR controls showed amplification and low inter-plate variability. *HPRT*1, *RPL*13*A* and *GAPDH* were selected as the most

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stable housekeeping genes and used for normalization. *B2M* was revealed as the most unsuitable reference gene due to high variation in its expression (see Supplementary information).

3.2. Interleukin-11, Interleukin-6 and Tumor Necrosis Factor as transcriptomic biomarkers

Transcriptional differences were considered between responders and non-responders in the target genes *IL11*, *IL6* and *TNF* both before and after treatment with escitalopram. Binary logistic regressions were used to assess differences between responders and non-responders to treatment with escitalopram at each week. Each target gene's relative expression was considered as a covariate, along with age, sex, center of treatment and baseline MADRS scores.

Relative expression of *IL11* did not show any significant differences between responders and non-responders at baseline (β=0.006, S.E.=0.044, p=0.891; Figure 1), but there was a significant difference in *IL11* relative expression between responders and non-responders after treatment with escitalopram (week 8, β=0.725, S.E.=0.359, p=0.044; Figure 1). Responders showed approximately 45% lower expression than non-responders after treatment with escitalopram for eight weeks.

IL6 relative expression did not show any significant differences between responders and non-responders either at baseline (week 0; β=−0.139, S.E.=0.366, p=0.704), or after treatment with escitalopram (week 8; β=0.141, S.E.=0.397, p=0.723).

Relative expression of *TNF* showed significant differences between responders and non-responders at baseline (β=1.601, S.E.=0.752, p=0.033; Figure 2), with *TNF* showing approximately 17% lower expression in responders compared to non-responders. Similarly, after treatment with escitalopram (week 8), there were significant differences between responders and non-responders, with *TNF* showing approximately 30% lower expression in responders (β=1.721, S.E.=0.724, p=0.017; Figure 2).

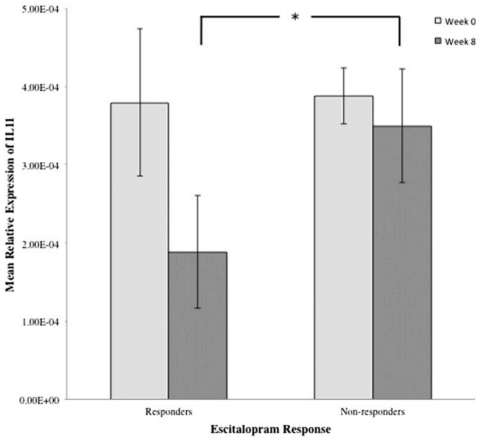

Figure 1 Bar graph showing the mean relative expression of the target gene *IL11* in responder and non-responder groups. The relative expression values ($2^{-\Delta C_t}$) are shown on the *y*-axis, and clinical responder status is shown on the *x*-axis. The light gray bars represent gene expression at baseline (week 0), and the dark gray bars represent gene expression after treatment with escitalopram (week 8), with error bars representing the S.E.M. Binary logistic regressions were used to assess differences between responders and non-responders at each week, covarying for age, sex, center of treatment and baseline MADRS scores. Expression differences of $p \leq 0.05$ are denoted by ∗.

3.3. Allele-specific (rs1126757) expression differences in IL11

Previous reports in the whole of the GENDEP cohort suggest that carriers of at least one A allele in rs1126757 predicts better treatment response compared to those homozygous for the G allele (GG genotype). Within our subset, we had the following genotype frequencies: AA (n=8), AG (n=26), GG (n=11). Using binary logistic regressions with response as the dependent variable, we assessed whether expression differences between responders and non-responders in *IL11* were being driven by the presence or absence of the A allele at rs1126757, with age, sex, center of recruitment and baseline MADRS as covariates. Likewise, in this subset of GENDEP samples, we found that carriers of the A allele (AA/AG genotypes) responded significantly better than individuals homozygous for the G allele (β=2.390, S.E.=1.05, p=0.024). Using univariate linear regressions we then investigated whether relative expression of *IL11* at each week was being driven by genotype. We considered *IL11* expression as the dependent variable, center, sex and *IL11* genotype as fixed factors, and age as a covariate. Genotype did not significantly drive expression at baseline (F=0.37; p=0.896), but did after eight weeks of treatment with escitalopram (F=8.93; p=0.008). The univariate linear model revealed that genotype accounts for 31% of the variance in the relative expression of *IL11* after escitalopram treatment. Furthermore, those with the A allele showed approximately 50% lower expression of *IL11* in response to escitalopram compared to those homozygous for the G allele (Figure 3).

3.4. Transcriptomic biomarkers in the wider inflammatory cytokine pathway

A total of 67 target genes (and five housekeeping genes) out of 86 on the qPCR array were sufficiently detectable in patient blood samples, according to our set criteria. Binary logistic regression revealed that there were no significant expression differences between responders and non-responders either before or after treatment, after correction for multiple testing. The most significant expression difference based on uncorrected p-values was in *IL10RA* at baseline, and *B2M* after escitalopram treatment (see Table 1). Those genes with uncorrected p-values of $p \leq 0.05$ were inputted into Ingenuity Pathway Analysis along with *TNF* and *IL11* to identify any specific part of the

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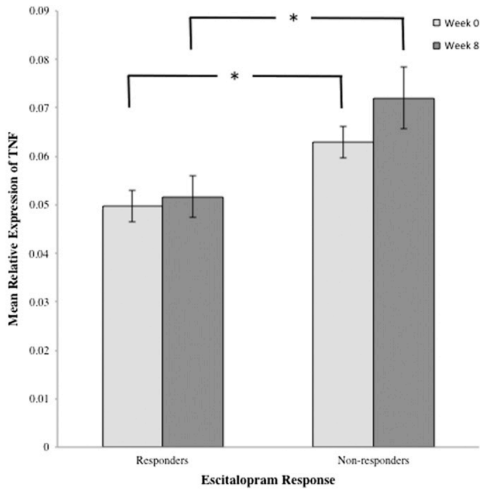

Figure 2 Bar graph showing the mean relative expression of the target gene *TNF* in responder and non-responder groups. The relative expression values ($2^{-\Delta C_t}$) are shown on the *y*-axis, and clinical responder status is shown on the *x*-axis. The light gray bars represent gene expression at baseline (week 0), and the dark gray bars represent gene expression after treatment with escitalopram (week 8), with error bars representing the S.E.M. Binary logistic regressions were used to assess differences between responders and non-responders at each week, covarying for age, sex, center of treatment and baseline MADRS scores. Expression differences of $p \leq 0.05$ are denoted by $*$.

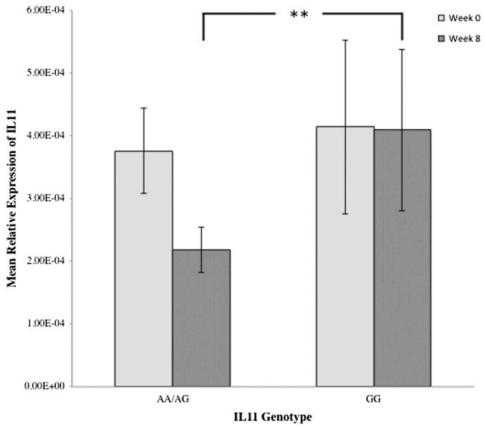

Figure 3 Bar graph showing the mean relative expression ($2^{-\Delta C_t}$) of the target gene *IL11* in two different genotype groups for the SNP rs1126757, those homozygous for the G allele (GG genotype) or those with the A allele (AA/AG genotypes). The relative expression values ($2^{-\Delta C_t}$) are shown on the *y*-axis, and the genotype groups on the *x*-axis. The light gray bars represent gene expression at baseline (week 0), and the dark gray bars represent gene expression after treatment with escitalopram (week 8), with error bars representing the S.E.M. Univariate linear regressions were used to assess whether the expression of IL11 at each week was predicted by genotype group, covarying for age, sex and center of treatment. Genotype-specific expression differences of $p \leq 0.01$ are denoted by $**$.

inflammatory cytokine pathway which may be mediating response. Interestingly, at both weeks, genes were revealed to be targets of, or targeted by, TNF. We additionally tested whether expression of these targets correlated to the expression of *TNF* shown in Figure 4 (see Table S3 for more details).

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Table 1 Results of the binary logistic regressions comparing expression differences between responders and non-responders both at baseline (week 0) and after escitalopram treatment (week 8); these include *B*-values, standard error of mean (S.E.M.) and *p*-values. Gene symbols represent the genes in the inflammatory cytokine pathway present on the array.

Gene	*Week 0*			*Week 8*		
	***B*-value**	**S.E.M**	***p*-value**	**B-value**	**S.E.M**	**p-value**
IL6	−0.139	0.366	0.704	0.141	0.397	0.723
IL11	0.006	0.044	0.891	0.725	0.359	**0.044**
TNF	1.601	0.752	**0.033**	1.721	0.724	**0.017**
ABCF1	1.447	0.740	**0.050**	−1.043	0.750	0.164
BCL6	0.607	0.448	0.176	0.360	0.418	0.389
C3	0.349	0.363	0.336	0.586	0.335	0.080
C4A	0.265	0.287	0.356	−0.530	0.271	0.846
C5	1.408	0.592	**0.017**	0.509	0.510	0.319
CCL1	−0.525	0.702	0.454	1.715	1.333	0.198
CCL2	0.375	0.294	0.202	0.319	0.260	0.221
CCL3	0.677	0.458	0.140	0.654	0.377	0.083
CCL4	1.485	0.700	**0.034**	1.472	0.667	**0.027**
CCL5	1.035	0.553	0.061	0.547	0.424	0.198
CCL18	0.441	0.562	0.432	0.788	0.943	0.403
CCL19	0.008	0.345	0.981	−0.368	0.411	0.370
CCL20	0.201	0.307	0.512	0.015	0.212	0.945
CCL23	−0.006	0.287	0.983	0.275	0.267	0.303
CCL24	0.579	0.337	0.086	1.051	0.491	**0.032**
CCL25	0.173	0.181	0.339	0.245	0.212	0.248
CCL26	0.467	0.410	0.254	1.233	0.546	**0.024**
CCL8	0.870	0.587	0.138	0.353	0.438	0.421
CCR1	0.645	0.411	0.117	0.440	0.355	0.216
CCR2	−0.099	0.469	0.834	0.099	0.410	0.810
CCR3	−0.345	0.305	0.911	0.092	0.280	0.743
CCR4	0.278	0.362	0.442	0.316	0.314	0.315
CCR5	0.994	0.529	0.060	0.874	0.457	0.056
CCR6	0.701	0.585	0.231	0.911	0.584	0.119
CCR9	−1.396	0.551	**0.011**	0.526	0.426	0.217
CEBPB	−0.250	0.331	0.450	−0.032	0.342	0.925
CX3CR1	0.127	0.389	0.745	0.351	0.379	0.355
CXCL1	−0.851	0.504	0.092	0.009	0.329	0.979
CXCL2	0.505	0.333	0.129	0.351	0.299	0.240
CXCL3	0.230	0.323	0.467	0.178	0.412	0.666
CXCL5	0.185	0.278	0.505	0.172	0.303	0.571
CXCL6	−0.300	0.277	0.279	−0.131	0.217	0.544
CXCL9	0.300	0.339	0.929	0.097	0.223	0.665
CXCL10	−0.408	0.293	0.164	0.056	0.271	0.837
CXCL11	1.527	0.741	**0.039**	0.726	0.663	0.273
CXCL12	−0.151	0.422	0.719	1.629	0.422	0.203
CXCL13	0.717	0.466	0.124	0.301	0.312	0.334
IL10	0.331	0.313	0.291	0.029	0.231	0.899
IL10RA	2.522	0.912	**0.006**	0.963	0.506	0.057
IL10RB	0.250	0.487	0.607	0.180	0.378	0.634
IL13RA1	0.215	0.389	0.580	0.400	0.414	0.334
IL17C	0.071	0.326	0.827	0.495	0.374	0.186
IL1A	0.450	0.249	0.070	0.115	0.271	0.671
IL1B	0.101	0.342	0.767	0.491	0.390	0.208
IL1F6	0.274	0.410	0.503	−0.022	0.284	0.939
IL1F7	0.010	0.260	0.969	0.542	0.278	0.052
IL1R1	−0.004	0.305	0.990	−0.226	0.287	0.430
IL1RN	−0.137	0.206	0.508	−0.053	0.374	0.887
IL22	−0.192	0.241	0.424	−0.299	0.252	0.236
IL5	0.310	0.293	0.290	0.702	0.384	0.068
IL5RA	0.020	0.317	0.949	0.096	0.300	0.750

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Table 1 (*continued*)

Gene	*Week 0*			*Week 8*		
	B-value	S.E.M	*p*-value	B-value	S.E.M	p-value
IL8	−0.215	0.331	0.516	−0.064	0.276	0.817
IL8RA	0.313	0.450	0.487	0.085	0.381	0.823
IL8RB	0.592	0.440	0.179	0.533	0.405	0.189
LTA	1.563	0.726	**0.031**	1.514	0.725	**0.037**
LTB	0.657	0.626	0.294	1.421	0.789	0.072
LTB4R	0.214	0.422	0.612	0.381	0.489	0.436
MIF	0.235	0.654	0.719	1.983	0.897	**0.027**
SCYE1	−0.397	0.480	0.408	0.337	0.521	0.517
SPP1	0.040	0.170	0.814	0.176	0.211	0.696
CD40LG	0.954	0.598	0.110	0.383	0.681	0.574
TOLLIP	0.105	0.560	0.852	−0.605	0.630	0.337
XCR1	0.316	0.371	0.395	0.330	0.369	0.372
B2M	0.000	0.567	0.998	2.053	0.736	**0.005**

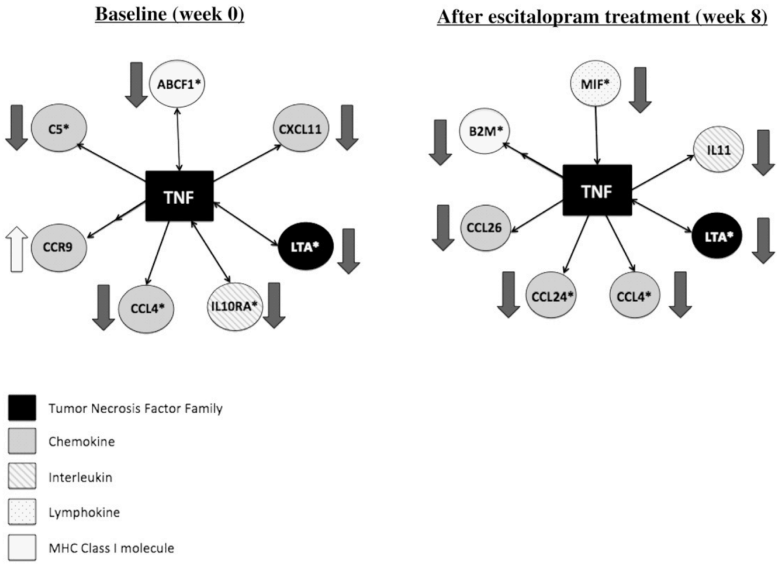

Figure 4 This diagram depicts the top gene expression differences in the inflammatory cytokine pathway between responders and non-responders to escitalopram at baseline (left) and after eight weeks of escitalopram treatment (right). Top gene expression differences were selected based on binary logistic regressions assessing expression differences between responders and non-responders at baseline, covarying for age, sex, center of treatment and baseline MADRS scores, producing uncorrected *p*-values of $p<0.05$. All genes were shown to be targets of, or targeted by, TNF. Single arrows between TNF and its targets represent direct targets, whereas two arrows represent an intermediate molecule linking TNF with its target. Genes are colored according to the subgroup of inflammatory cytokine in which they belong. Thick arrows next to each gene show the direction of expression in responders compared to non-responders, i.e. downward arrows represent lower expression of that gene in responders. Those genes which correlate to the expression of TNF ($p<0.05$) are marked with a $*$.

4. Discussion

This study aimed to identify transcriptomic biomarkers for clinical response to the SSRI antidepressant escitalopram in the inflammatory cytokine pathway. The first part of the study investigated three candidate genes, interleukin 11 (*IL11*), interleukin 6 (*IL6*) and tumor necrosis factor (*TNF*), which all have strong prior evidence supporting their

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involvement in mediating response to escitalopram. The second part of the study considered transcriptomic differences between responders and non-responders in the wider inflammatory cytokine pathway in a hypothesis-free manner.

A genome-wide association study attempting to identify genetic variants underlying clinical treatment response to escitalopram found a single nucleotide polymorphism (rs1126757) in the *IL11* gene with suggestive genome-wide significance (Uher et al., 2010). Consequently, the transcription of *IL11* was considered in this study. We found transcription of *IL11* after escitalopram treatment predicted clinical response but did not at baseline (see Figure 1). We further investigated the role of the GWAS suggestive rs1126757 and whether it could be driving the expression differences seen between responders and non-responders. The GWAS revealed that carriers of the A allele (AG/AA genotypes) were more likely to respond to escitalopram than GG genotype individuals; an effect also observed in the current subsample, see Section 3.3 (Results). Prior to treatment, genotype did not significantly predict expression of *IL11* but did after treatment with escitalopram, with carriers of the A allele showing a significant reduction in expression relative to the GG genotype (see Figure 3). Consequently, the results reveal an allele-specific decrease in *IL11* expression in response to escitalopram that is subsequently associated with clinical response. Whilst the prediction of clinical outcome after eight weeks of treatment may not be useful in personalizing treatment, our results shed light on a potential biological mechanism of antidepressants. For instance, it is known that IL11 inhibits serotonin signaling in the brain by causing raphe neurons to produce acetylcholine instead of serotonin (Rudge et al., 1996). Consequently, reduced *IL11* transcription in response to escitalopram may disinhibit this mechanism and thus promote serotonin signaling in the brain. In addition, decreased *IL11* expression in responders aligns with previous evidence suggesting clinical response to antidepressants is associated with a decrease in proinflammatory cytokines (Tuglu et al., 2003).

IL6 was investigated for its role in antidepressant response due to previous studies finding differences at the protein level between depressed patients and non-depressed controls (Maes et al., 1997) and a SNP that had been implicated in escitalopram response (Uher et al., 2010). However, in our study *IL6* did not show any significant expression differences between responders and non-responders either at baseline or after treatment with escitalopram. Thus it may be possible that the major effect of IL6 occurs as a result of protein interactions and not at the level of the transcriptome in relation to antidepressant response.

TNF has frequently been implicated in both the pathophysiology and treatment of depression. We found that individuals who fail to respond to treatment with escitalopram have increased *TNF* expression both at baseline and after eight weeks of treatment (see Figure 2). Previous findings at the protein level showed serum levels of TNF to be higher in individuals with depression (Mikova et al., 2001; Yang et al., 2007) and that higher serum levels of TNF predict non-responsiveness to antidepressants (Tuglu et al., 2003) including escitalopram (Eller et al., 2008). These findings are in agreement with the results shown here at the transcriptional level and suggest that differences at the protein level are not simply a case of protein post-processing differences but differences that manifest at the level of the gene.

Functionally, TNF has been shown to enhance serotonin transporter function in human placental cell lines (Mossner et al., 1998), rat neuronal cell lines and in mouse brain (Zhu et al., 2006). As such, it is possible that different expression levels of *TNF* between responders and non-responders may relate to differences in the functionality of the serotonin transporter, the target of selective serotonin reuptake inhibitors such as escitalopram. Furthermore, TNF has also been shown to induce the enzyme indoleamine 2, 3-dioxygenase (IDO) which breaks down the precursor to serotonin (Miller et al., 2008). Thus, it is possible that higher transcription of *TNF* and subsequently higher protein levels inhibits serotonin synthesis. TNF has also been shown to have direct inhibitory effects on neurogenesis in the hippocampus (Monje et al., 2003; Iosif et al., 2003), a believed therapeutic effect of antidepressants. Consequently, higher levels of *TNF* amongst non-responders may prevent neurogenesis thus hindering a potential therapeutic effect of the antidepressant. As far as these authors are aware, this is the first study to identify *TNF* as a transcriptomic biomarker for response to an antidepressant. Future studies should focus on the contributions of genetic, epigenetic and environmental processes in mediating these expression differences.

Within the hypothesis-free component of the study, there were no significant differences between responders and non-responders in any of the genes after correction for multiple testing. However, pathway analysis revealed that both at baseline, and after escitalopram treatment, genes showing differences (based on uncorrected p-values of $p \leq 0.05$) between responders and non-responders were targets of, or themselves a target of, TNF (see Figure 4). One of these TNF targets includes another member of the TNF family, the closely related lymphotoxin alpha gene (*LTA*; formerly known as *TNF-*β) which similarly acts as a biomarker for escitalopram response both at baseline and after escitalopram treatment. This lends further support for the importance of the TNF family as transcriptomic biomarkers for escitalopram response.

Our pathway results corroborate previous reports at the protein level which show inflammatory cytokines to be lower in responders compared to non-responders (Eller et al., 2008; Tuglu et al., 2003). Furthermore, it suggests that *TNF* and its targets may constitute a select hub within the wider inflammatory cytokine pathway involved in mediating response to escitalopram.

The results from the current study add to growing support that augmentation therapies targeting inflammatory cytokines may successfully increase the rate of responders to antidepressant treatment. In particular clinical reports suggest that the use of TNF antagonists such as infliximab may have useful applications in the treatment of depression (Maas et al., 2010), particularly in treatment resistant depression (Miller, 2012).

The authors acknowledge the limitations of the current study which include the use of a relatively small sample size, and the use of cut-offs which attempt to dichotomize

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clinical response. Future studies should attempt to replicate findings detailed here in larger samples with sufficient power to investigate change in response in a linear manner. Another limitation of this study is that blood cell counts were not performed. This would have ascertained whether whole blood gene expression was being affected by different quantities of cell types. Previous research on the 'macrophage hypothesis' of depression might argue that a cell count is particularly pertinent (Smith, 1991), and as such would be useful to perform in future studies. However the use of blood over other tissues such as brain is clearly a strength in this study, as blood represents an easily accessible, renewable biomarker resource.

The current study draws strength from its utilization of stringent clinical methodology, sensitive and accurately normalized measures of gene expression, and use of appropriate covariates in analyses. The study reports a number of important and novel findings, namely that mRNA expression levels of *TNF* may be suitable as a biomarker for clinical response to the selective serotonin reuptake inhibitor escitalopram.

Pathway analysis revealed that genes interacting with TNF may also act as biomarkers, suggesting a select hub within the wider inflammatory cytokine pathway may be mediating response to the antidepressant escitalopram. *IL11* shows differential expression between responders and non-responders but only after treatment with escitalopram. Moreover, expression differences in *IL11* were found to be mediated by allele-specific expression changes relating to the previously reported pharmacogenetic GWAS hit rs1126757. This study provides strong support for the role of genetic differences in the inflammatory cytokine pathway in mediating response to escitalopram, and demonstrates the possible utility of transcriptomic biomarkers in this pathway as predictors of clinical response.

Note added in the proofs

During the submission of this manuscript, another study by Cattaneo et al. (2012) was published which also reports *TNF* as a predictor of antidepressant response. The current study utilizes an independent non-overlapping set of samples.

Role of funding source

The GENDEP project was funded by the European Commission Framework 6 Grant, EC Contract Ref. LSHB-CT-2003-503428. Lundbeck provided both nortriptyline and escitalopram free of charge for the GENDEP project. The current study was financially supported by the NIHR Biomedical Research Centre for Mental Health at the South London and Maudsley NHS Foundation Trust and Institute of Psychiatry, Kings College London under the Grant BRC 08/09PP. The funders had no role in the design and conduct of the study, in data collection, analysis, interpretation or writing the report. Dr. Aitchison holds an Alberta Centennial Addiction & Mental Health Research Chair funded by the Government of Alberta. Timothy R. Powell is funded by a Medical Research Council PhD studentship.

Contributors

Authors D'Souza, Craig, Danese, Lewis, Uher, Farmer, Aitchison and McGuffin were involved in the design of the GENDEP project, the design of the current study and in writing the grant which funded the study. Breen, Lawrence and Heffernan were involved in RNA blood sample extractions. Tansey, Price and Schalkwyk provided guidance in the analysis of the data, with Tansey also contributing to the preparation of the manuscript. Powell was involved in the majority of the laboratory work, in data collection, analysis and in writing the manuscript.

Conflict of interest

Powell, Schalkwyk, Heffernan, Breen, Lawrence, Price, Craig, Danese, Lewis, Uher, Tansey and D'Souza have no competing interests. Farmer, Aitchison and McGuffin have received consultancy fees and honoraria for participating in expert panels from pharmaceutical companies, including Lundbeck and GlaxoSmithKline.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2012.09.009.

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Chapter 5

Investigating the utility of DNA methylation in interleukin-11 as a predictor of antidepressant response

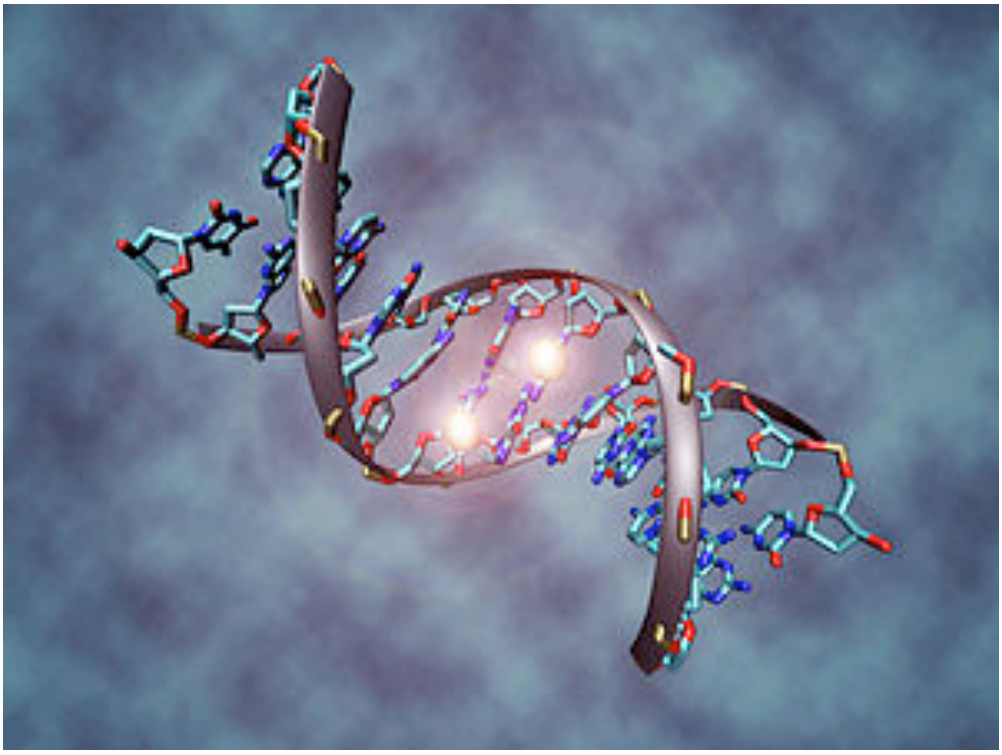

Figure 5.0: This image shows a DNA molecule that is methylated on both strands on the centre cytosine. Image created by Christoph Bock (Max Planck Institute).

5.0 Notes

This chapter consists of a manuscript published in *Translational Psychiatry* in September 2013. This work follows results described in Chapter 4. For more information on previous work relating to epigenetics and MDD, see *Section 1.1.7.* For consistency with the wider thesis, *Introduction* in this manuscript is referred to as Section 5.1 elsewhere, *Figure 1* within the manuscript will be referred to as Figure 5.1, *Table 1* will be referred to as Table 5.1 etc.

The method for DNA methylation quantification employed in this chapter was developed by Elrich et al. 2005. Briefly, the method uses bisulfite treatment to produce DNA methylation-dependent sequence changes, converting non-methylated cytosine nucleotides to thymine. These cytosine/thymine variations appear as guanine/adenine changes on the reverse strand, which result in a mass difference of 16 Da per CpG site in the cleavage products that have CpG sites. The mass difference is then detected by the MassARRAY system contributing to differential signal pattern, which allows us to quantify methylation at CpG sites.

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ORIGINAL ARTICLE

DNA methylation in interleukin-11 predicts clinical response to antidepressants in GENDEP

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Transcriptional differences in interleukin-11 (*IL11*) after antidepressant treatment have been found to correspond to clinical response in major depressive disorder (MDD) patients. Expression differences were partly mediated by a single-nucleotide polymorphism (rs1126757), identified as a predictor of antidepressant response as part of a genome-wide association study. Here we attempt to identify whether DNA methylation, another baseline factor known to affect transcription factor binding, might also predict antidepressant response, using samples collected from the Genome-based Therapeutic Drugs for Depression project (GENDEP). DNA samples from 113 MDD individuals from the GENDEP project, who were treated with either escitalopram ($n = 80$) or nortriptyline ($n = 33$) for 12 weeks, were randomly selected. Percentage change in Montgomery–Åsberg Depression Rating Scale scores between baseline and week 12 were utilized as our measure of antidepressant response. The Sequenom EpiTYPER platform was used to assess DNA methylation across the only CpG island located in the *IL11* gene. Regression analyses were then used to explore the relationship between CpG unit methylation and antidepressant response. We identified a CpG unit predictor of general antidepressant response, a drug by CpG unit interaction predictor of response, and a CpG unit by rs1126757 interaction predictor of antidepressant response. The current study is the first to investigate the potential utility of pharmaco-epigenetic biomarkers for the prediction of antidepressant response. Our results suggest that DNA methylation in *IL11* might be useful in identifying those patients likely to respond to antidepressants, and if so, the best drug suited to each individual.

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INTRODUCTION

Major depressive disorder (MDD) is predicted to be the second leading cause of disability by 2020.[1] Antidepressants are currently the first line of treatment for MDD, but around two-thirds of patients fail to respond to the first antidepressant prescribed, and a third fail to respond to multiple antidepressant treatments.[2] Studies have attempted to establish biomarkers to predict response to antidepressant medication and to personalize treatment. Genetic biomarkers have been investigated as predictors of clinical outcome; however, results from large-scale pharmacogenetic studies have mostly been unsuccessful in identifying genes that are robustly associated with clinical antidepressant response.[3–7]

However, recent evidence draws further support to results from one genome-wide association study performed in the Genome-based Therapeutic Drugs for Depression project (GENDEP), which identified a single-nucleotide polymorphism (SNP) (rs1126757) in interleukin-11 (*IL11*) that predicted response to the selective serotonin reuptake inhibitor escitalopram.[4] Further investigation of *IL11* at the transcriptional level found it to be expressed at a lower level in responders compared with that in non-responders after treatment with escitalopram, but not before the initiation of escitalopram treatment.[8] Gene expression differences after treatment were partially mediated by rs1126757, implicating rs1126757 as a treatment-emergent expression quantitative trait locus.[8]

Similarly to expression quantitative trait loci, DNA methylation can also affect transcription factor binding and moderate gene expression changes, and DNA methylation has been linked to the pathophysiology of mood disorders.[9,10] Subsequently, in this study we aimed to investigate whether baseline DNA methylation in *IL11* could be used to predict antidepressant response.

The current study used blood samples collected from 113 individuals diagnosed with MDD as part of the GENDEP project. We attempted to identify: (1) whether there are DNA methylation predictors of general antidepressant response (independent of drug or genotype); (2) whether there are differential DNA methylation predictors, which could be used to predict whether an individual is more likely to respond to the antidepressant escitalopram or nortriptyline; and (3) whether there is an interaction between rs1126757 genotype and DNA methylation, which predicts response to antidepressants.

MATERIALS AND METHODS

Clinical sample

Patient samples were taken from the GENDEP project, which has been described in detail elsewhere.[11] Briefly, GENDEP is a 12-week, partially randomized, open-label pharmacogenetic study with two active treatment arms. A total of 868 treatment-seeking adults (men: $n = 321$; women: $n = 547$) with MDD of at least moderate severity according to the ICD-10 or DSM-IV criteria were recruited from 9 European centers. Patients were

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aged 19–72 years and were of Caucasian European parentage. Diagnoses were established using the semi-structured Schedules for Clinical Assessment in Neuropsychiatry interview.[12] Exclusion criteria were personal and family history of schizophrenia or bipolar disorder, current substance dependence, or whether participants had previously taken both of the drugs and demonstrated treatment resistance. Eligible participants were allocated to treatment with either the selective serotonin reuptake inhibitor escitalopram (total $n=394$) or the noradrenaline reuptake inhibitor nortriptyline (total $n=312$), which differed by antidepressant mechanisms of action.[13] Patients with no contraindications were randomly allocated to a flexible-dosage of nortriptyline (50–150 mg daily) or escitalopram (10–30 mg daily) for 12 weeks. Patients with contraindications for one drug were offered the other. The GENDEP project was approved by ethics boards of participating centers, and all participants provided a written consent after the procedures were explained. GENDEP is registered at EudraCT (No.2004-001723-38, https://eudract.ema.europa.eu/) and ISRCTN (No. 03693000, http://www.controlled-trials.com).

Participants were assessed for severity of depressive symptoms by using the clinician-rated Montgomery-Åsberg Depression Rating Scale (MADRS) on a weekly basis.[14] Previous work found that the MADRS scores were the most sensitive, clinically representative and internally consistent scores for depression symptom changes in GENDEP, and, consequently, is the measure we use in this study.[15]

A subset of 113 individuals (males $n=46$; females $n=67$; age 40.3 years ± 11.7 years) were randomly selected among patient samples, who had complete clinical data for the full 12 weeks of treatment and had genome-wide association study genotype data. All patients had a diagnosis of moderate to severe MDD, with a baseline severity of 27.7 ± 4.8 (average MADRS score, s.d.). Individuals were treated with either the antidepressant escitalopram ($n=80$) or nortriptyline ($n=33$). Fewer than 10% of individuals had previously taken an antidepressant on entering GENDEP and all individuals were drug-free for 2 weeks before the start of the study. Before this, patients had reported taking antidepressants ($n=10$), benzodiazepines ($n=35$) and hypnotics ($n=14$). The average duration of the current depressive episode in our sample was 21.3 ± 19.3 weeks (average duration, s.d.). Seventy-two of our patients had experienced a stressful life event within 6 months before entering the GENDEP study, as measured using the List of Threatening Experiences Questionnaire.[16] Percentage change in the MADRS score from baseline to week 12 was used as a measure of antidepressant response. Higher positive changes in the percentage MADRS represent better treatment response.

Experimental details

Genotyping. Patient blood samples were collected and stored in ethylenediaminetetraacetic acid (EDTA), after which DNA was extracted using a standard extraction procedure.[17] Genotype data were collected as part of a genome-wide association study.[4] Full details of genotyping and quality-control measures can be found elsewhere.[4] Briefly, samples were sent to the Centre National de Genotypage (Evry, France) and were genotyped using the Illumina Human 610-Quad BeadChips (Illumina, San Diego, CA, USA), which genotypes more than 600 000 SNPs. All 113 patients included in the current study passed the routine quality-control tests, which included removing individuals for ambiguous sex, abnormal heterozygosity, cryptic relatedness (up to third-degree relatives), genotyping incompleteness (<97% coverage) and non-white European admixture. Genotype data include the rs1126757 SNP. Genotype data for this SNP was extracted using PLINK.[18]

DNA methylation. All DNA samples were quantified and tested for purity using the Nanodrop ND1000 (Thermo Scientific, Wilmington, DE, USA). Previous quality-control measures were employed for the purpose of genotyping and DNA had been stored at −80 °C.

Four hundred nanograms of genomic DNA was treated with sodium bisulfite using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA, USA) following the standard manufacturer's protocol. *IL11* primer design was based on *in-silico* bisulfite-amplicon prediction using the Mass array package (Bioconductor, www.bioconductor.org) in R (http://www.R-project.org). Primers were designed to span the CpG island in *IL11* (chr 19: 55880511–55880989, Genome Reference Consortium GRCh37/USCS version hg19, University of California Santa Cruz Genome Browser). Forward primers consisted of the following sequence: 5′-GATGGAGTTGGAGG TTTTAAGTTTTA-3′. Reverse primers consisted of the following sequence 5′-ACCCATAACTCTACCCCTCTCC-3′. For each 10 μl reaction, the polymerase chain reaction mastermix consisted of the following: 1 μl 10 × buffer (Qiagen, Crawley, UK), 0.2 μl dNTPs (10 μM; Thermo Scientific, Northumberland, UK), 0.2 μl $MgCl_2$ (Thermo Scientific, UK), 0.1 μl HotStarTaq Polymerase (Qiagen), 1 μl *IL11* forward primer (Sigma-Aldrich, Poole, UK), 1 μl *IL11* reverse primer (Sigma-Aldrich), 2 μl DNA and 4.5 μl water. Thermal cycling conditions consisted of an initial enzyme activation stage (95 °C for 10 min); followed by 35 cycles of denaturation (95 °C for 30 s), hybridization (58 °C for 30 s) and extension (72 °C for 1 min); and a final single-extension step (72 °C for 4 min) and cool-down step (4 °C for 10 min).

Controls included both artificially hypermethylated DNA (positive control) and RNAse-free water (no template control). Polymerase chain reactions were performed in duplicate and the products were pooled together to reduce technical variation. DNA methylation was quantitatively assessed using the Sequenom EpiTYPER system (Sequenom, San Diego, CA, USA) as described previously.[19] Data generated from the EpiTYPER software were filtered using in-built quality-control parameters, and CpG units with low call rates (that is, <90% call rates) were removed.

Statistical analysis

Statistical analyses (i–iii) included age, sex, center of recruitment, baseline MADRS score and allocated antidepressant drug as covariates. For CpG units displaying non-normal distributions, we applied the square-root transformation.

(i) DNA methylation as a predictor of general antidepressant response. To investigate whether DNA methylation in *IL11* could be used as a predictor of general antidepressant response, we performed univariate linear regressions with percentage MADRS change as the dependent variable and CpG unit methylation included as a covariate.

(ii) Differential drug by DNA methylation predictors of antidepressant response. To assess whether CpG unit methylation might interact with our two antidepressant drugs to differentially predict an antidepressant response, we performed univariate linear regressions. Percentage MADRS change was selected as our dependent variable and covariates included CpG unit methylation and the interaction between the allocated antidepressant drug and the CpG unit methylation.

(iii) DNA methylation × rs1126757 predictors of antidepressant response. To investigate whether there was an interaction between rs1126757 and CpG unit methylation that could predict antidepressant response, we performed a univariate linear regression with percentage MADRS change as the dependent variable, and covariates including rs1126757 genotype, CpG unit methylation and the interaction between the CpG unit methylation and rs1126757.

(iv) Multiple testing correction. We entered all *P*-values generated from analyses i–iii into a single false discovery rate calculation to generate *q*-values. We achieved this using an online false discovery rate web-based

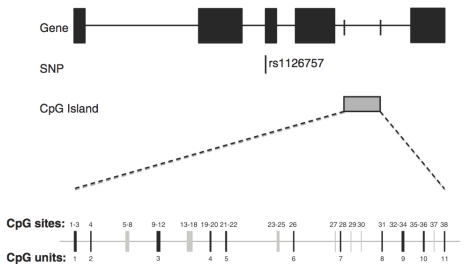

Figure 1. Schematic diagram of interleukin-11 (*IL11*) in a 5′ to 3′ direction, with the grey box showing the CpG island our assay covers and black boxes representing exons (top). Pictogram representing the individual CpG units within the CpG island, with black lines noting the CpG units adequately detected by the Sequenom and grey lines showing CpG units not assessed by this method (bottom).

tool available at http://www.sdmproject.com/utilities/?show=FDR. All *q*-values ⩽0.1 were considered to be true effects.

(v) Effects of medication, episode duration and recent stressful life events on DNA methylation. To assess whether previous medication use, the duration of the current depressive episode or the presence of a recent stressful life event might be driving any of our false discovery rate-significant predictors, we performed secondary analyses. We performed a univariate linear regression with CpG unit methylation as the dependent variable, and use of benzodiazepine, antidepressants, hypnotics and the presence or absence of a recent stressful life event were included as binary covariates, with episode duration (weeks) included as a continuous covariate.

RESULTS

Results from DNA methylation experiments revealed that all positive controls (hypermethylated DNA) showed greater than 85% detected levels of methylation and all no template controls (H_2O) showed 0% methylation. Eleven out of a possible 18 CpG units were adequately detected by the Sequenom and passed quality-control steps (Figure 1). Mean levels of the CpG unit methylation (%) and standard error (s.e.) at each of the 11 CpG units can be seen in Figure 2.

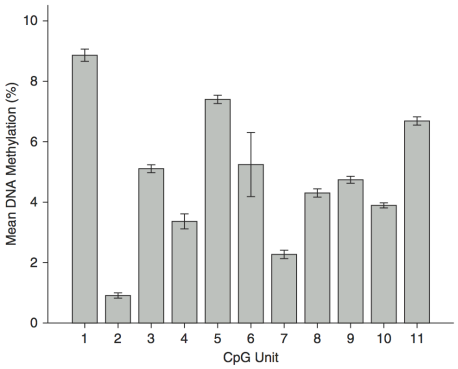

Figure 2. Bar graph showing mean percentage DNA methylation in our total sample at each of the 11 CpG units spanning the interleukin-11 (IL11) CpG island. CpG unit location is shown on the *x*-axis and methylation (%) is shown on the *y*-axis.

DNA methylation as a predictor of general antidepressant response

A univariate linear regression was performed to assess whether methylation levels at CpG units in *IL11* predicted response to either antidepressant medication. CpG unit 5 significantly predicted antidepressant response ($F = 8.429$, d.f. $= 1$, $\eta_p^2 = 0.082$, $P = 0.005$, $q = 0.055$; Table 1). Lower levels of DNA methylation at CpG unit 5 was associated with better response to antidepressants (Figure 3).

Differential DNA methylation by drug predictors of antidepressant response

Univariate linear regressions were performed to assess whether DNA methylation at any of the CpG units in *IL11* acted as a predictor of differential response. Methylation at CpG unit 4 significantly predicted differential response to treatment ($F = 8.412$, d.f. $= 1$, $\eta_p^2 = 0.083$, $P = 0.005$, $q = 0.055$; Table 1). Higher levels of DNA methylation at CpG unit 4 was associated with better response in individuals taking escitalopram, but was associated with worse response in those taking nortriptyline (Figure 4).

DNA methylation by rs1126757 interaction predictors of antidepressant response

A univariate linear regression was performed to test whether an interaction between methylation at any CpG unit and rs1126757 genotype predicted antidepressant response. An interaction between methylation at CpG unit 11 and rs1126757 significantly predicted response to treatment ($F = 6.821$, d.f. $= 2$, $\eta_p^2 = 0.131$, $P = 0.002$, $q = 0.055$; Table 1). Individuals homozygous for the G-allele (GG), who had higher levels of methylation at CpG unit 11, responded better to antidepressant treatment than those individuals homozygous for the A allele (AA), with no effect of DNA methylation observed in heterozygotes (AG) (Figure 5).

Testing for confounding factors

Linear regressions revealed that none of our possible confounding factors (previous medication use, duration of depressive episodes, or occurrence of a recent stressful life event) were driving DNA

Table 1. A summary of the results from the univariate linear regressions

	DNA methylation				*Drug × DNA methylation*				*Genotype × DNA methylation*			
CpG unit	*F*	*d.f*	P	q	*F*	*d.f*	P	q	*F*	*d.f*	P	q
1	0.002	1	0.965	0.966	0.097	1	0.757	0.961	0.784	2	0.459	0.854
2	0.784	1	0.378	0.854	1.672	1	0.199	0.854	0.742	2	0.479	0.854
3	0.058	1	0.810	0.966	0.051	1	0.821	0.966	0.080	2	0.924	0.966
4	1.438	1	0.234	0.854	8.412	1	**0.005**[a]	**0.055**	0.363	2	0.697	0.961
5	8.429	1	**0.005**[a]	**0.055**	2.477	1	0.119	0.854	0.135	2	0.874	0.966
6	0.853	1	0.358	0.854	0.109	1	0.742	0.961	0.034	2	0.966	0.966
7	0.327	1	0.569	0.854	0.327	1	0.569	0.854	0.711	2	0.494	0.854
8	0.407	1	0.525	0.854	0.407	1	0.525	0.854	1.911	2	0.154	0.854
9	0.566	1	0.454	0.854	1.533	1	0.219	0.854	0.594	2	0.554	0.854
10	0.525	1	0.470	0.854	0.525	1	0.470	0.854	0.756	2	0.472	0.854
11	0.010	1	0.920	0.966	0.131	1	0.718	0.961	6.821	2	**0.002**[a]	**0.055**

Results include an F statistic, d.f., *P*-values and *q*-values.
A summary of the results from the univariate linear regressions in which we tested whether (from left to right) DNA methylation, drug by DNA methylation interactions, or rs1126757 genotype by DNA methylation interactions in each of the 11 CpG units could predict antidepressant response. [a]Significant *P*-values ($P \leqslant 0.005$) are highlighted in bold.

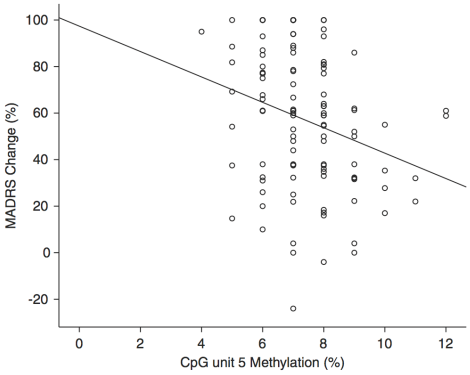

Figure 3. Scatter plot of the relationship between DNA methylation at CpG unit 5 (*x*-axis) and percentage Montgomery-Åsberg Depression Rating Scale (MADRS) change (*y*-axis). Line represents the line of best fit. DNA methylation at CpG unit 5 significantly predicted percentage MADRS change in our model ($P = 0.005$).

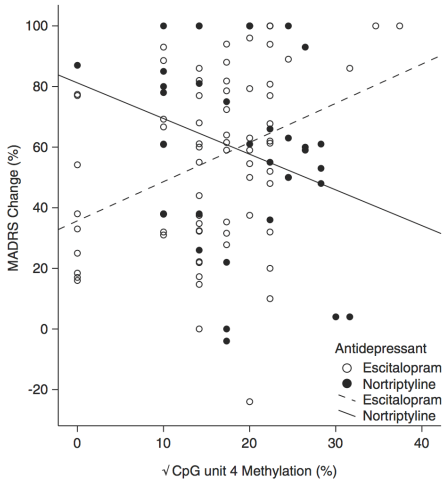

Figure 4. Scatter plot of the relationship between percentage DNA methylation at CpG unit 4 (*x*-axis) and percentage Montgomery-Åsberg Depression Rating Scale (MADRS) change (*y*-axis). Lines represent line of best fit for each drug group. DNA methylation at CpG unit 4 was found to significantly interact with the drug type to predict percentage MADRS change in our model ($P = 0.005$).

methylation in any of our three FDR-significant DNA methylation predictors.

DISCUSSION

MDD is becoming an increasing global concern, creating an urgent need for effective treatment.[1,2] With high interindividual

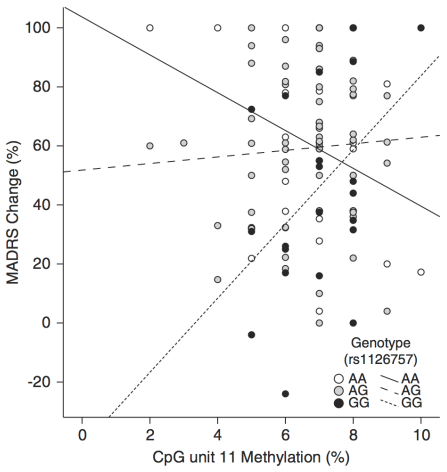

Figure 5. Scatter plot of the relationship between percentage DNA methylation at CpG unit 11 (*x*-axis) and percentage Montgomery-Åsberg Depression Rating Scale (MADRS) change (*y*-axis). Data points and lines of best fit correspond to different genotypes of the genome-wide association study single-nucleotide polymorphism, rs1126757. DNA methylation at CpG unit 11 was found to significantly interact with rs1126757 to predict percentage MADRS change in our model ($P = 0.002$).

variation in treatment response to antidepressants, the search for biomarkers aims to personalize therapy and improve upon our current 'trial and error' method of treatment selection.[3] Genetic, proteomic and, recently, transcriptomic biomarker studies have attempted to identify predictors of antidepressant response, with varied success.[3–8,20] Our previous work identified a treatment-emergent expression quantitative trait locus (rs1126757) in *IL11*, which predicted response to the antidepressant escitalopram.[8] The SNP driving the observed transcriptional differences had previously been identified as a predictor of response to escitalopram as part of a genome-wide association study.[4,8] Subsequently, we hypothesized that other baseline factors with the potential to affect transcription factor binding and gene expression changes in *IL11* might also predict antidepressant response. Here we explored the potential utility of DNA methylation in *IL11* as a baseline predictor of antidepressant response.

The results detailed here are the first to demonstrate the potential use of pharmaco-epigenetic biomarkers as the baseline predictors of antidepressant response. Our results suggest that CpG unit-specific DNA methylation in *IL11* could be used to predict whether an individual is likely to respond to antidepressants (see Figure 3) and, if they are, the type of drug best suited to each individual (see Figure 4). The interaction between genotype and DNA methylation also reveals the importance of integrating genotype and methylation data in search of molecular biomarkers for antidepressant response (see Figure 5).

IL11 has previously been found to induce potent inhibitory effects on serotonin signaling.[21] Consequently, it adds to a growing number of functionally relevant cytokines, which have been previously associated with antidepressant response (for example, tumor necrosis factor and IL-6).[4,8,20,22] It also further supports suggestions that augmentation therapies targeting the cytokines might be useful in improving response to antidepressants.[23–25]

The cause of our observed DNA methylation differences remains unclear, but based on our results they are unlikely to be related to episode duration, current medication use or experiences of a recent stressful life event. In future studies, it would be interesting to test whether early stressful life events might predict DNA methylation in *IL11*, as early life stressors are known modulators of the methylome, affect the levels of inflammatory markers and act as a risk factor for MDD.[26–29]

Despite the promising results detailed here, the study has three main limitations. First, the differences in DNA methylation observed are small and CpG unit-specific; hence, further studies are required to determine whether these differences are biologically meaningful. Second, although blood has useful biomarker properties (e.g., it is renewable, peripherally accessible and has access to the brain tissue), further work is still required to understand how DNA methylation differences in the blood might relate to the differences in the brain. Third, our method could not detect DNA methylation at all CpG sites, and in some cases it used averages across neighboring CpG sites to form CpG units (see Figure 1).

In conclusion, results presented here are the first to demonstrate the potential clinical utility of DNA methylation biomarkers as predictors of antidepressant response. Future studies are further needed to replicate these findings and validate the relationship between site-specific methylation in *IL11* and antidepressant response.

CONFLICT OF INTEREST

PM has received consultancy fees and honoraria for participating in expert panels from pharmaceutical companies, including Lundbeck and GlaxoSmithKline. All other authors declare no conflict of interest.

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Chapter 6

Investigating the effects of escitalopram treatment on transcription within the inflammatory cytokine pathway

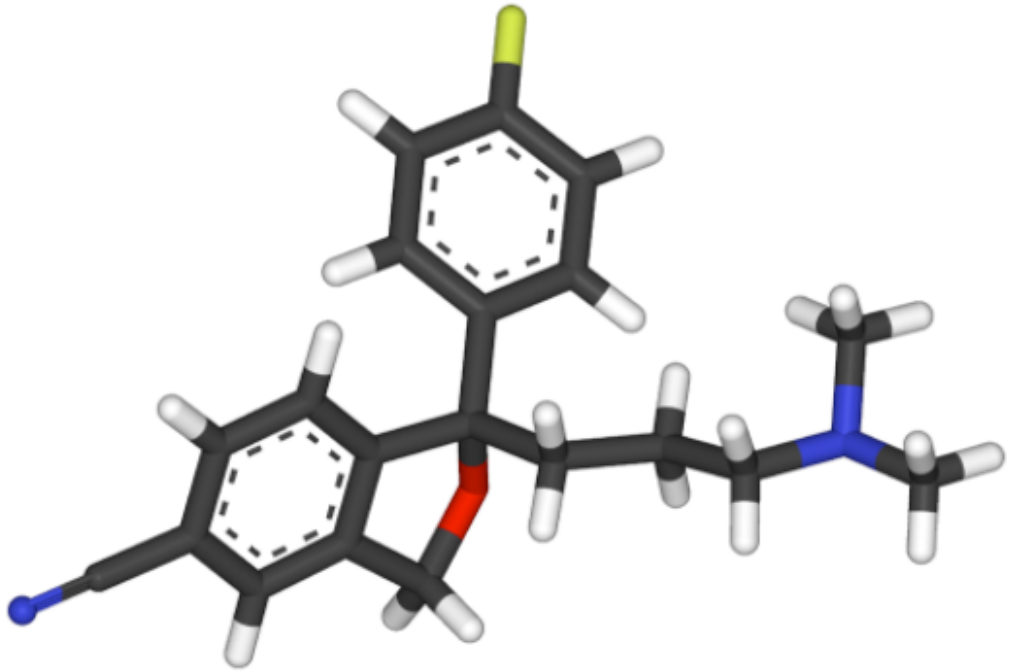

Figure 6.0: A computer generated image of the chemical structure of escitalopram [(S)-1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile].

6.0 Notes

This chapter consists of a manuscript published in *Journal of Psychopharmacology* published in July 2013. For more information on escitalopram see *Section 1.3.1.1.* For more information on antidepressant-induced transcriptional changes and treatment-emergent biomarkers for antidepressant response see Section *1.5.1.3.* For consistency with the wider thesis, *Introduction* in this manuscript is referred to as Section 6.1 elsewhere, *Figure 1* within the manuscript will be referred to as Figure 6.1, *Table 1* will be referred to as Table 6.1 etc.

Original Paper

ATP-binding cassette sub-family F member 1 (ABCF1) is identified as a putative therapeutic target of escitalopram in the inflammatory cytokine pathway

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Abstract
The inflammatory cytokine pathway may be a potential therapeutic target for major depressive disorder (MDD). Previous reports suggest that antidepressants have anti-inflammatory properties and can cause a reduction in proinflammatory cytokines. Recent evidence suggests this might be mediated at the level of the transcriptome. The current study investigated the transcription of 86 genes in the inflammatory cytokine pathway both at baseline and after eight weeks of escitalopram treatment in MDD patients who were either clinical responders (n=25) or non-responders (n=21), using a subset of samples in the Genome-Based Therapeutic Drugs for Depression project (GENDEP). Changes in expression between baseline and eight weeks of treatment were assessed using two-tailed t-tests. To establish if any significant expression changes related to clinical response, the magnitude of the relative expression change between baseline and eight weeks of treatment was established and binary logistic regressions were used to compare differences between responders and non-responders. ATP-binding cassette sub-family F member 1 (*ABCF1*), a translational regulator of the inflammatory cytokine pathway showed a significant increase in expression after escitalopram treatment which was significantly greater in responders compared to non-responders, suggesting that *ABCF1* may play a role in mediating antidepressant response.

Keywords
Selective serotonin reuptake inhibitor, pharmacogenetic, inflammation, transcription

Introduction

Major depressive disorder (MDD) is a complex heterogeneous disorder that is equally complex to treat. Imbalances in the monoamine neurotransmitter systems are believed to relate to the causes of MDD, and it is the system primarily targeted by most antidepressants (Charney, 1998). However, only one-third of patients respond to the first antidepressant they are prescribed, and one-third fail to respond to any form of antidepressant (Gibson et al., 2010). Consequently, other systems may be playing a role in moderating antidepressant response.

The inflammatory cytokine pathway is one system that shows potent interactions with the monoamines and has been identified as a possible moderator of antidepressant therapy (Maes et al., 2011; Powell et al., 2012, Rudge et al., 1996). Proinflammatory cytokines have been shown to increase in response to stress (a predictor of depressive episodes), be present at higher concentrations amongst MDD patients relative to controls, and be at higher baseline levels amongst non-responders to antidepressant therapy (Eller et al., 2008; Hiles et al., 2012; Kendler et al., 1999; Maes et al., 1998). Interestingly, antidepressants are known to have anti-inflammatory properties (Abdel-Salam et al., 2003; Roumestan et al., 2007), which are mediated through both a reduction in proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-6 (IL6) and interleukin 1-β (IL1-B), and an increase in anti-inflammatory cytokines such as interleukin-10 (IL-10) (Kenis and Maes, 2002; Kubera et al., 2001). Thus, these changes that occur after antidepressant treatment, are thought to 'correct' the increased levels of proinflammatory cytokines reportedly present amongst MDD patients, implicating the inflammatory cytokines as potential targets or moderators of antidepressant therapy.

The notion that cytokines act as moderators of antidepressant therapy is further supported by reports at the genetic and transcriptional levels. For instance, studies have shown that single nucleotide polymorphisms in *IL-6* and interleukin-11 (*IL-11*), and baseline transcriptional levels of *TNF*, predict clinical responsiveness to the selective serotonin reuptake inhibitor (SSRI) escitalopram (Powell et al., 2012; Uher et al., 2010;). Furthermore, escitalopram has been found to induce numerous changes to gene transcription, which include targets of cytokines in the mouse brain (Malki et al., 2012), and changes to *IL6, IL-1B* and macrophage migration

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inhibitory factor (*MIF*) transcription in MDD patient blood (Cattaneo et al., 2012). Interestingly, changes to *IL6* transcription in response to antidepressants has also been found to relate to clinical response in MDD patients (Cattaneo et al., 2012).

Consequently, the current literature suggests that abnormal levels of inflammatory cytokines present amongst MDD patients are 'corrected' by antidepressant treatment, which may arise through changes in gene transcription. Furthermore, the magnitude of transcriptional changes in cytokine genes in response to antidepressant treatment may be the key process moderating clinical antidepressant response. However, no studies have yet attempted to replicate these findings reported at the transcriptional level, nor has there been an extensive exploration of the relationship between antidepressant-induced transcriptional changes in the wider inflammatory cytokine pathway and its relationship to clinical response.

The current study utilized blood samples from the pharmacogenetic Genome-Based Therapeutic Drugs for Depression project (GENDEP). The aim was to investigate the effects of the SSRI escitalopram on gene transcription in 86 genes within the inflammatory cytokine pathway by comparing transcription of genes at baseline and after eight weeks of treatment with escitalopram. These 86 genes include members of the complement component class of cytokines, the chemokines and their receptors, the interleukins and their receptors, and other inflammatory-related genes, in an attempt to identify whether particular gene families are more affected by escitalopram than others. Furthermore, we determined whether any significant gene expression changes that occurred after treatment with escitalopram were related to clinical response.

Methods

Sample

The current study utilized samples from the wider GENDEP project which has been described in detail elsewhere previously (Uher et al., 2009). Briefly, GENDEP is a 12-week partially randomized open label pharmacogenetic study with two active treatment arms. A total of 868 treatment seeking adults of Caucasian European parentage (men: n=321; women: n=547; age 19–72 years) with major depressive disorder of at least moderate severity according to International Statistical Classification of Diseases 10 (ICD-10) or Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria were recruited from nine European centers. Diagnoses were established using the semistructured Schedules for Clinical Assessment in Neuropsychiatry interview (Wing et al., 1998). Exclusion criteria were personal and/or family history of schizophrenia or bipolar disorder and/or current substance dependence. Eligible patients with no contraindications were randomly allocated to a flexible-dosage of a norepinephrine reuptake inhibitor (nortriptyline 50–150 mg daily) or a serotonin reuptake inhibitor (escitalopram 10–30 mg daily) for 12 weeks. Patients with contraindications for one drug were offered the other. The GENDEP project was approved by ethics boards of participating centers, and all participants provided written informed consent. The current study focuses on the effects of the SSRI escitalopram. This drug was chosen because previous studies in GENDEP have linked this drug more strongly to the inflammatory cytokines as putative mediators of clinical response (Powell et al., 2012; Uher et al., 2010).

Table 1. Summary of patient characteristics.

Patient characteristic	Responders	Non-responders	Total sample
Sample number (n)	25	21	46
Age (mean, SD)	41.80±12.87	43.57±12.08	42.61±12.41
Males (n)	14	6	20
Females (n)	11	15	26
MADRS (mean, SD)	29.28±4.99	30.38±5.49	29.78±5.19
BMI (mean, SD)	25.25±3.59	26.77±3.76	25.94±3.70
Smoker (n)	3	3	6
Antidepressant (n)	5	7	12
Benzodiazepine (n)	9	12	21
Hypnotics (n)	3	2	5

BMI: body mass index; MADRS: Montgomery-Åsberg Depression Rating Scale; SD: standard deviation.

Participants were assessed for severity of depressive symptoms on a weekly basis using the clinician-rated Montgomery-Åsberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979). Percentage change in MADRS scores from baseline to week 8 were used to dichotomize patients into responders or non-responders. For the purposes of this study, those patients with a percentage MADRS change of greater than 50% were considered responders, whereas those with a percentage MADRS change of less than 50% were considered non-responders. A subset from GENDEP totaling 46 patients was selected and investigated in the current study. These 46 individuals were randomly selected from a group of patients who fulfilled the following four criteria: (a) patients were treated with escitalopram, (b) patients had complete clinical data for the full 12 week treatment period, (c) blood was collected from these patients both at baseline and at week 8, (d) the subset contained approximately equal numbers of responders (n=25) and non-responders (n=21). Patient characteristics are summarized in Table 1.

RNA extraction

Ten mL of blood was collected in PAXgene tubes (PreAnalytiX, Hombrechtikon, Switzerland) at baseline and again after eight weeks of treatment with escitalopram, and frozen at –80°C. Prior to the start of gene expression studies, PAXgene tubes were allowed to thaw for 12 h at room temperature. RNA extraction was performed using the Qiagen PAXgene Blood miRNA Kit (PreAnalytiX) following the standard manufacturer's protocol. The purity and quantity of RNA was measured using the Nanodrop, ND1000 (Thermoscientific, Wilmington, Delaware, USA), and RNA integrity numbers (RINs) were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Berkshire, UK). All samples had 260/280 ratios of between 1.9–2.3 and the average RIN was 8±1.5.

Quantitative polymerase chain reaction *(PCR) protocol*

Reagents used for the quantitative PCR (qPCR) were manufactured by SABiosciences (Frederick, Maryland, USA). 1 µg of total RNA and the SABiosciences RT² HT First Strand Kit were used to

generate cDNA samples following the manufacturer's protocols. Generated cDNA samples were then stored at –20°C prior to use. Customized 384-well qPCR arrays were designed and contained lyophilized primers for the 84 genes listed in the commercially available Human Inflammatory Cytokines & Receptors PCR Array (SABiosciences), with the addition of gene primers for *IL11* and *IL6*. Three housekeeping genes were selected for normalization based on geNorm and Normfinder analyses previously reported in Powell et al. (2012). These genes included: hypoxanthine phosphoribosyltransferse (*HPRT1*), ribosomal protein L13a (*RPL13A*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Each qPCR array was designed to analyze four samples simultaneously. Each array contained cDNA from one responder and one non-responder, both at baseline and after eight weeks of escitalopram treatment. Each qPCR array contained the following controls: human genomic DNA control (gDNA), reverse transcription control (RTC) and a positive PCR control (PPC). The qPCR reaction consisted of: 550 μL of 2X SABiosciences RT^2 qPCR Master Mix (SYBR green), 102 μL of diluted synthesized cDNA and 448 μL RNAse free water, with a total volume of 1100 μL for each patient sample. 10 μL of this mix was then pipetted to each well on the array.

The qPCR reactions were performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, California, USA). Thermal cycling conditions consisted of an enzyme activation stage (95°C for 10 min), followed by 40 cycles of a denaturation stage (95°C for 15 s) and a hybridization and extension stage (65°C for 1 min). The software program SDS 2.3 (Applied Biosystems) generated cycle threshold values (C_t) from the data collected.

Statistical analysis

C_t values of greater than 37 were removed and excluded from further analysis as such high C_t values are indicative of very low expression levels. If, as a result of data removal, a gene showed missing data for more than a third of the total patient sample, that gene was excluded from further analysis. The relative expression of target genes was derived using the formula $2^{-\Delta Ct}$, where delta C_t (ΔC_t) is the difference between the C_t of the target gene and the mean C_t of the selected reference genes (Livak and Schmittgen, 2001). Differences in the relative expression of genes before and after treatment were determined using two-tailed paired student *t*-tests. False discovery rate (FDR) was used to detect true expression changes after escitalopram treatment accounting for multiple testing, where q-values of q<0.1 were considered to be true effects. Any FDR-significant expression changes underwent a further statistical test to determine if the expression changes were driven by clinical responder status. To investigate this we determined relative expression change, which was calculated by subtracting ΔCt (week 8) from ΔCt (week 0) to generate ΔΔCt values. ΔΔCt were subsequently used in regression analyses and $2^{-\Delta\Delta Ct}$ was used to generate bar charts (Livak and Schmittgen, 2001). Using binary logistic regressions with responder status (responder/non-responder) as the dependent variable, we examined whether change in expression over the eight weeks predicted response, covarying for age, sex, centre of treatment and baseline MADRS score. We additionally repeated the same analysis to determine if any significant gene expression changes that predicted response after 8 weeks of treatment also continued to predict response at the end the GENDEP trial (week 12). Responder status at week 12 was calculated in the same way as responder status at week 8 (using percentage changes in MADRS scores), but instead using MADRS data collected at week 12.

For any significant expression changes, we further performed linear regressions to assess whether previous medication use, smoking status, or body mass index might be driving expression differences. All statistical analyses were performed using SPSS Version 15 (SPSS Inc., Chicago, Illinois, USA).

Results

All qPCR plates passed quality control checks and all positive controls showed amplification and low inter-plate variability.

Transcriptional changes in the inflammatory cytokine pathway in response to escitalopram. A total of 57 genes out of the 86 genes on the array were sufficiently detectable in patient blood samples, according to our set criteria. We performed paired *t*-tests to assess transcriptional differences between baseline and eight weeks of treatment with escitalopram (see Table 2). Three genes produced significant uncorrected *p*-values, including those for ATP-binding cassette sub-family F member 1 (*ABCF1; t*=–3.457 (45), *p*=0.0009), complement component 5 (*C5; t*=–2.439 (45), *p*=0.019) and toll interacting protein (*TOLLIP; t*=2.012 (45), *p*=0.05). Only *ABCF1* remained significant after the FDR method of multiple correction (q<0.03, Figure 1).

ABCF1 *expression change and its relationship to clinical response.* To determine if expression changes in *ABCF1* might be driving clinical response, we performed a binary logistic regression with responder/non-responder as the dependent variable, *ABCF1* expression change as an independent variable, and age, sex, centre of treatment and baseline MADRS scores as covariates. *ABCF1* expression changes significantly predicted clinical response (β=–1.641, standard error (SE)=0.730, *p*=0.025), with responders showing a greater expression change than non-responders (Figure 2). Additionally, expression changes between baseline and week 8 continued to significantly predict responder status at the end of the GENDEP trial, after 12 weeks of antidepressant treatment; (β=–2.077, SE=0.786, *p*=0.008): with responders still showing significantly higher levels of expression change. Expression changes in *ABCF1* were found not to be influenced by any previously used medications, smoker status or body mass index based on linear regression results.

Discussion

Previous research has linked antidepressant response to protein and transcriptional changes to members of the inflammatory cytokine pathway. The current study aimed to investigate the effects of the SSRI antidepressant escitalopram on the transcription of 86 genes in the inflammatory cytokine pathway using whole blood collected from MDD patients. We further considered whether such transcriptional changes were related to the therapeutic response.

This study revealed that the majority of the inflammatory cytokines showed no transcriptional changes in response to escitalopram treatment. There were no significant decreases in the transcription of the proinflammatory cytokines *TNF, IL-6, IL-1B* or

Table 2. Results of the paired *t*-test investigating differences at baseline and after eight weeks of treatment with escitalopram: these include *t*-values, degrees of freedom (df), *p*-values and *q*-values. Gene symbols and the gene family to which they belong to are indicated. Significant *p*-values (uncorrected) and *q*-values are shown in bold.

Gene family	Gene	*t*	df	*p*-value	*q*-value
Complement	C3	−0.789	30	0.436	0.398
	C4A	−1.526	45	0.134	0.338
	C5	−2.439	45	**0.019**	0.258
Chemokine	CCL2	0.542	45	0.590	0.398
	CCL3	−1.433	45	0.159	0.359
	CCL4	−0.924	45	0.360	0.398
	CCL5	−0.406	45	0.686	0.398
	CCL20	0.693	34	0.493	0.398
	CCL23	0.853	45	0.398	0.386
	CCL24	−0.653	45	0.517	0.398
	CCL25	−0.909	45	0.368	0.386
	CXCL1	0.314	45	0.755	0.398
	CXCL2	0.936	38	0.355	0.386
	CXCL5	0.77	45	0.446	0.398
	CXCL6	0.584	45	0.562	0.398
	CXCL9	−1.518	41	0.137	0.338
	CXCL10	1.156	43	0.254	0.386
Chemokine	CCR1	−0.961	45	0.342	0.386
receptor	CCR2	−1.916	45	0.062	0.338
	CCR3	−0.367	45	0.715	0.398
	CCR4	−0.664	45	0.510	0.398
	CCR5	−1.007	45	0.319	0.386
	CCR6	1.091	45	0.281	0.386
	CCR9	0.457	45	0.650	0.398
	CXCR1	0.371	45	0.731	0.398
	XCR1	−1.64	45	0.108	0.338
Interleukin	IL1A	1.387	33	0.175	0.365
	IL1B	1.562	45	0.125	0.338
	IL1F6	−0.058	33	0.954	0.462
	IL1F7	−0.666	41	0.509	0.398
	IL5	0.564	45	0.576	0.398
	IL6	0.356	38	0.724	0.398
	IL8	0.081	45	0.936	0.462
	IL10	−0.17	45	0.865	0.434
	IL17C	−0.365	44	0.717	0.398
	IL22	0.465	32	0.645	0.398
Interleukin	IL1R1	−0.517	45	0.608	0.398
receptor	IL1RN	1.00	45	0.322	0.386
	IL5RA	−1.846	45	0.071	0.338
	IL8RA	1.733	45	0.090	0.338
	IL8RB	0.342	44	0.734	0.398
	IL10RA	−0.221	44	0.826	0.423
	IL10RB	1.01	45	0.318	0.386
	IL13RA1	0.376	45	0.709	0.398
Tumor necrosis	TNF	−1.763	45	0.085	0.338
factor	LTA	1.04	45	0.304	0.386
	LTB	0.449	45	0.656	0.398
	LTB4R	0.304	45	0.763	0.398
	CD40LG	−0.988	45	0.329	0.386
Other inflammatory	ABCF1	−3.547	45	**0.0009**	**0.024**
regulators	BCL6	−0.927	45	0.359	0.386
	CEBPB	0.875	45	0.386	0.386
	MIF	0.015	45	0.988	0.470

Table 2. (Continued)

Gene family	Gene	*t*	df	*p*-value	*q*-value
	SCYE1	1.231	45	0.225	0.386
	SPP1	0.859	44	0.395	0.386
	TOLLIP	2.012	45	**0.050**	0.338

ABCF1: ATP-binding cassette, sub-family F (GCN20), member 1; BCL6: B-cell CLL/lymphoma 6; C3: Complement component 3; C4A: Complement component 4A; C5: Complement component 5; CCL2: Chemokine (C-C motif) ligand 2; CCL20: Chemokine (C-C motif) ligand 20; CCL23: Chemokine (C-C motif) ligand 23; CCL24: Chemokine (C-C motif) ligand 24; CCL25: Chemokine (C-C motif) ligand 25; CCL3: Chemokine (C-C motif) ligand 3; CCL4: Chemokine (C-C motif) ligand 4; CCL5: Chemokine (C-C motif) ligand 5; CCR1: Chemokine (C-C motif) receptor 1; CCR2: Chemokine (C-C motif) receptor 2; CCR3: Chemokine (C-C motif) receptor 3; CCR4: Chemokine (C-C motif) receptor 4; CCR5: Chemokine (C-C motif) receptor 5; CCR6: Chemokine (C-C motif) receptor 6; CCR9: Chemokine (C-C motif) receptor 9; CEBPB: CCAAT/enhancer binding protein (C/EBP), beta; CXCL1: Chemokine (C-X-C motif) ligand 1; CXCL10: Chemokine (C-X-C motif) ligand 10; CXCL2: Chemokine (C-X-C motif) ligand 2; CXCL5: Chemokine (C-X-C motif) ligand 5; CXCL6: Chemokine (C-X-C motif) ligand 6; CXCL9: Chemokine (C-X-C motif) ligand 9; IL10: Interleukin 10; IL10RA: Interleukin 10 receptor, alpha; IL10RB: Interleukin 10 receptor, beta; IL13RA1: Interleukin 13 receptor, alpha 1; IL17C: Interleukin 17C; IL1A: Interleukin 1, alpha; IL1B: Interleukin 1, beta; IL1F6: Interleukin 1 family, member 6; IL1F7: Interleukin 1 family, member 7; IL1R1: Interleukin 1 receptor, type 1; IL1RN: Interleukin 1 receptor antagonist; IL22: Interleukin 22; IL5: Interleukin 5; IL5RA: Interleukin 5 receptor, alpha; IL8: Interleukin 8; IL8RA: Interleukin 8 receptor, alpha; IL8RB: Interleukin 8 receptor, alpha; LTA: Lymphotoxin alpha; LTB: Lymphotoxin beta; LTB4R: Leukotriene B4 receptor; MIF: Macrophage migration inhibitory factor; SCYE1: Small inducible cytokine subfamily E, member 1; SPP1: Secreted phosphoprotein 1; TNF: Tumor necrosis factor; CD40LG: CD40 ligand; TOLLIP: Toll interacting protein; XCR1: Chemokine (C motif) receptor 1.

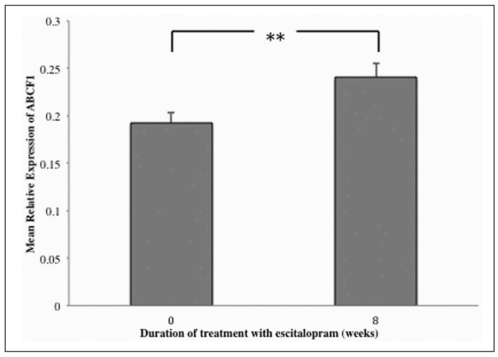

Figure 1. Bar graph showing the mean relative expression of the target gene ATP-binding cassette sub-family F member 1 (*ABCF1*) both at baseline (week 0) and after escitalopram treatment (week 8). The relative expression values ($2^{-\Delta Ct}$) are shown on the *y*-axis, and duration of escitalopram treatment (weeks) is shown on the *x*-axis. The bars represent mean relative expression at each week, with error bars representing the standard error. Paired *t*-tests were used to assess differences between week 0 and week 8. Expression differences of $p \leq 0.01$ are denoted by **.

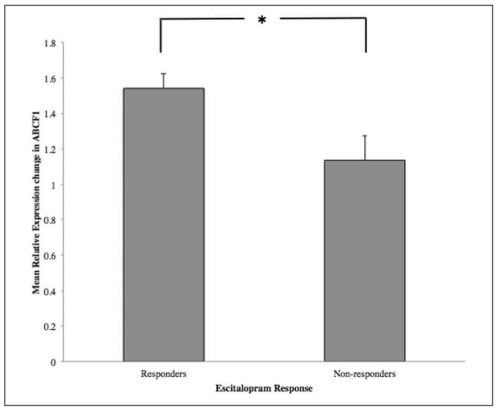

Figure 2. Bar graph showing the mean relative expression change in the target gene ATP-binding cassette sub-family F member 1 (*ABCF1*) after eight weeks of treatment with escitalopram. Relative expression change $2^{-\Delta\Delta Ct}$ is shown on the *y*-axis, and clinical responder status is shown on the *x*-axis. Error bars represent the standard error. Binary logistic regressions were used to assess the expression change difference between responders and non-responders, covarying for age, sex, center of treatment and baseline Montgomery-Åsberg Depression Rating Scale (MADRS) scores. Expression differences of $p \leq 0.05$ are denoted by *.

MIF previously shown to change at the protein or transcriptional level in response to antidepressants (Bah et al., 2011; Cattaneo et al., 2012; Hannestad et al., 2011). Neither were there increases in the transcription of anti-inflammatory cytokines such as *IL-10*, previously shown to increase in response to antidepressants at the protein level (Maes et al., 1999). These results suggest that SSRIs may not target the inflammatory cytokines at the level of the transcriptome. The previous report, which revealed significant effects of antidepressants on the transcription of cytokines, investigated patients treated with either an SSRI or a tricyclic antidepressant (Cattaneo et al., 2012). Thus, it could be that the transcriptional effects of antidepressants on cytokines previously reported might be more strongly driven by the tricyclic antidepressant class, as opposed to the SSRI class.

Interestingly, one gene, *ABCF1*, did show a significant increase in transcription after escitalopram treatment (p=0.0009; see Figure 1). Lowered expression of *ABCF1* has previously been reported amongst schizophrenia patients relative to controls (De Jong et al., 2012). In relation to MDD, *ABCF1* has been identified as a putative baseline transcriptomic predictor of clinical response to escitalopram (Powell et al., 2012), and the broader superfamily of ATP-binding cassette (ABC) transporters have been associated with MDD, antidepressant response and remission (Dong et al., 2009; Singh et al., 2012).

The ABCF1 protein itself functions as a 'translational regulator', facilitating protein translation through its interaction with eukaryotic initiation factor 2 (eIF2) (Tyzack et al., 2010). Particularly, ABCF1 is thought to regulate the translation of

inflammatory cytokines and has been shown to both regulate, and be regulated by, TNF (Richard et al., 1998). A recent study found that heterozygote ABCF1 +/– knock-out mice have significantly decreased levels of TNF in blood serum compared to wild-type controls. Furthermore, splenic macrophages extracted from these knock-out mice produced significantly less TNF and IL-6 in response to lipopolysaccharide treatment compared to cells extracted from wild-type controls (Wilcox, 2011). This suggests that ABCF1 may negatively regulate the translation of TNF and IL6, two proinflammatory cytokines shown to be increased in MDD patients and which have been previously reported to decrease on antidepressant treatment (Miller et al., 2009).

We additionally found that the magnitude of the transcriptional increase of *ABCF1* was significantly greater in clinical responders to escitalopram compared to non-responders (see Figure 2), and continued to predict responder status after 12 weeks of treatment with escitalopram. If ABCF1 is indeed a negative regulator of pro-inflammatory cytokine translation, then this would corroborate previous reports that a reduction in proinflammatory cytokines at the protein level is associated with response to SSRIs (Hannestead et al., 2011). Our results suggest that *ABCF1*, a key regulator in the production of inflammatory cytokines, shows a significant change in response to escitalopram at the level of the transcriptome, which is consequently related to therapeutic efficacy. This suggests that changes in the transcriptional rate of *ABCF1* over the course of treatment with escitalopram may be important for successful clinical response, and should be investigated as a potential target for augmentation therapies.

Despite the potentially important results reported here, the current study is limited in three main ways. Firstly, the study utilizes a relatively small sample size and uses categories in this sample to form responder/non-responder groups. Secondly, although we attempted to co-vary for possible confounding factors, we did not have information about the time of day in which blood was collected, which could potentially influence cytokine expression. Thirdly, without cell count information we cannot determine exactly which blood cells are being affected by escitalopram, nor can we tell whether expression changes in blood reflect what is happening in the brain. Nevertheless, the use of blood does provide potentially useful biomarker properties for monitoring treatment success, both near the start of treatment and throughout the course of treatment.

To conclude, the current study revealed that the translational regulator *ABCF1* is a target of the antidepressant escitalopram. Transcription of *ABCF1* increased in response to the treatment with escitalopram. The magnitude of this transcriptional change was related to the therapeutic efficacy of escitalopram, with responders showing a greater increase in transcription compared to non-responders. Future studies should attempt to replicate findings reported here in a larger sample, and investigate change in response in a linear manner. Additionally, it would be of interest to investigate the role of functional genetic variants and epigenetic modifications in the *ABCF1* gene to assess if these account for the differences in transcriptional change between responders and non-responders to escitalopram treatment, and thus whether they could be utilized as baseline predictors of response.

Conflict of interest

G Breen, I Craig, T Powell, L Schalkwyk, U D'Souza, K Tansey and R Uher have no competing interests. A Farmer and P McGuffin have received consultancy fees and honoraria for participating in expert panels from pharmaceutical companies, including Lundbeck and GlaxoSmithKline.

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Chapter 7

Conclusions and Future Directions

Figure 7.1: 'Teen Depression', an illustration by Robert Carter.

7.1 Summary of findings from this thesis

The main aims of this thesis were to identify novel gene-environment interactions contributing to MDD, to search for molecular biomarkers which might be used to aid clinical diagnosis and treatment selection for MDD, and to investigate the molecular effects of antidepressant treatment. Our first aim was addressed in Chapter 2, where we attempted to identify potentially novel gene-environment interactions, which might increase an individual's risk to MDD. We achieved this by investigating transcriptomic changes in the hypothalamus in response to maternal separation, using two inbred strains of mice; one of which showed long-lasting alterations in HPA-reactivity (C57BL/6J) and another which did not show changes in HPA-reactivity (DBA/2J). The aim was to model an early life stress, analogous to childhood neglect, in both mouse strains and investigate whether stress by strain interactions present at the level of the transcriptome in mouse could be used to prioritise the search for gene-environment interactions for MDD in humans. Our top stress x strain interaction in the mouse transcriptome was in *Terc,* which codes for the RNA component of the enzyme telomerase, used in the extension of telomeres. Telomeres have previously been found to be shorter amongst adults who have experienced early childhood neglect, and amongst mood disorder patients (Tyrka et al., 2010; Simon et al., 2006; Hartmann et al., 2010; Elvsashagen et al., 2011; Wikgren et al., 2012). Consequently, differential changes in *Terc* expression could offer a mechanism through which early stresses, such as neglect, evoke long-lasting alterations in telomere length. We further found an interaction between a SNP downstream of *TERC* (rs10936599) and severity of childhood emotional neglect which predicted MDD

case/control status in a human clinical cohort. As rs10936599 has previously been identified as the strongest genetic predictor of telomere length (Codd et al., 2013), it further supports the notion that molecular differences in *TERC* interacting with childhood emotional neglect may be responsible for differential telomere length, and is subsequently associated with MDD case/control status.

Our second aim was addressed in Chapter 3, where we attempted to identify molecular biomarkers to aid clinical diagnosis of MDD. Specifically we aimed to investigate whether transcriptomic biomarkers within the inflammatory cytokine pathway could be used to differentiate between control subjects, BPD patients and MDD patients. This study revealed that specific differences in the transcription of the chemokines *CCL24* and *CCR6* could differentiate MDD patients from BPD patients and control subjects. This subsequently suggests that disorder-specific differences in the transcription of chemokines might be useful clinically in differentiating MDD patients from BPD patients, helping to prevent misdiagnosis and the prescription of inappropriate pharmacotherapies.

Our third aim was to identify molecular predictors of antidepressant response and this was addressed in Chapters 4 and 5. In Chapter 4 we found that the transcription of *TNF* and its targets in the inflammatory cytokine pathway predicted clinical response to the SSRI antidepressant escitalopram. We further identified a treatment-emergent expression quantitative trait locus in *IL11*; whereby a SNP in *IL11* (rs1126757) identified as the best predictor of escitalopram response from a GWAS was associated with differential expression changes in response to the drug which corresponded with clinical response. In Chapter 5 we investigated whether DNA methylation in *IL11*, another baseline molecular factor known to affect gene expression, might also predict

antidepressant response or interact with rs1126757 to predict response. We found CpG-unit specific methylation differences within *IL11* which: predicted general antidepressant response; differentially predicted response to two different antidepressants with differing mechanisms of action (escitalopram and nortriptyline); interacted with rs1126757 to predict antidepressant response. Consequently, Chapter 4 details one of the first studies to identify transcriptomic predictors of antidepressant response, and Chapter 5 details the first study to identify putative baseline epigenetic predictors of antidepressant response.

Our final aim was addressed in Chapter 6 where we investigated whether the SSRI escitalopram induces changes to the transcription of genes within the inflammatory cytokine pathway, and whether such changes related to clinical response. We found significant increases in the transcription of *ABCF1* in response to escitalopram, with changes corresponding to clinical response. As ABCF1 is a translational regulator of the inflammatory cytokines, changes to its transcription may represent the mechanism through which antidepressants evoke changes to cytokine protein levels (e.g. Kenis & Maes, 2002). These results are the first to suggest that *ABCF1* may act as a novel therapeutic target of escitalopram.

7.2 Implications of Research Findings

Findings from Chapter 2 suggest that the maternal separation protocol in animal models might be useful in identifying novel susceptibility loci for MDD. Our findings specifically have potentially important implications for the prevention of MDD in a particular subgroup of individuals. Our results suggest that genetic

polymorphisms within *TERC,* with the ability to affect telomerase activity and telomere length, interact with childhood emotional neglect to predispose to MDD in adulthood. If our findings were replicated, and it was found that changes in *TERC* expression relate to a causative pathway of MDD, prevention strategies could be created. For instance, drugs, diet or exercise regimes which increase telomerase activity could be developed and prescribed to those with the risk-allele who have experienced childhood neglect in order to reduce their chances of developing MDD.

Results from Chapter 3 have two main implications. First, they suggest that transcriptional differences in the chemokines *CCL24* and *CCR6* could aid clinical diagnosis of MDD, differentiating MDD suffers from BPD individuals and healthy control subjects. If replicated, this could have important clinical implications, helping to improve diagnosis from the outset and prevent the prescription of inappropriate medication. Secondly, it may suggest that these two chemokines are specifically related to the pathophysiology of MDD and subsequently may represent important novel drug targets.

Results reported in Chapters 4 and 5, if replicated, could be used to improve treatment selection for MDD patients. We find evidence that molecular biomarkers in blood may be utilised as clinical predictors of antidepressant response. As only one-third of patients respond to the first antidepressant prescribed (Gibson et al., 2010), our findings have potentially very important implications in improving treatment response from the outset. The results in Chapter 5 are the first to find baseline epigenetic predictors of antidepressant response, providing the first preliminary evidence that pharmaco-epigenetics may be important in MDD treatment. In addition to finding epigenetic predictors of

general antidepressant response, we also identified differences in CpG-unit methylation which differentially predicted response to two antidepressants of different mechanisms of action. Consequently, if replicated, this could be utilised clinically to establish which type of antidepressant is best suited to each individual as quickly as possible, and it could reduce the costs associated with ineffective treatment.

Based on findings reported in Chapters 4 and 5, drugs targeting either TNF or IL11 may prove useful as adjuvant therapies for MDD treatment. In fact, a recent study suggests that adjuvant treatment with the TNF antagonist infliximab may help to improve antidepressant response (Raison et al., 2013). Results from Chapter 6 also suggest that ABCF1 may act as a therapeutic target of escitalopram and therefore drugs targeting ABCF1 may also be useful in treating MDD and improving response rates.

7.3 Future Directions

Based on the results detailed in this thesis, we can suggest numerous future research directions. Future studies should investigate the relationship between early life stress, *TERC* expression, telomerase activity and telomere length, with relation to MDD. This could best be achieved using a longitudinal study design in humans. Furthermore, a parallel mouse study could be used to establish whether differences in telomere length in a human peripheral tissue (e.g. lymphocytes) in response to early stress correspond with changes occurring in the brain.

In Chapter 3, we found transcriptional differences in *CCL24* and *CCR6* differentiated MDD patients from other subject groups. These findings require replication in a larger, drug-free patient cohort, and further analysis is required to determine how accurately these measures can be used to diagnose patients prospectively. As discussed in *Section 1.5*, biomarkers at different biological levels can potentially provide distinct, clinically useful information. Therefore, future studies should also establish whether genetic, epigenetic, or proteomic differences in *CCL24* or *CCR6* might also contain biomarker properties and whether differences at these molecular levels better differentiate MDD patients from other subject groups. Similarly, both TNF protein levels (e.g. Eller et al., 2008) and *TNF* transcription (reported in Chapter 4) have been found to predict response to escitalopram, thus future studies should evaluate which molecular measure more reliably captures response, and whether TNF specifically predicts response to the SSRI class of antidepressant only.

Based on results from Chapter 5, future research into antidepressant treatment response should consider the use of pharmaco-epigenetic biomarkers. As this study was the first of its kind, replication is essential. If *IL11* is confirmed as an epigenetic predictor of antidepressant response, it warrants the use of whole genome methods to assess whether other methylomic regions across the genome may also offer clinically useful biomarker information. Furthermore, as our results also revealed interactions between genomic factors and DNA methylation predicting antidepressant response, future statistical methods should be developed to incorporate genomic and methylomic data on a genome-wide scale.

7.4 Concluding Remarks

MDD is a complex heterogeneous disorder with both environmental and genetic risk factors. Through dissecting distinct aetiological pathways it may be possible to identify gene-environment interactions that contribute to the development of MDD. Furthermore, in the case of childhood neglect, it may be possible to model this in animals, and use molecular changes in mouse brain to prioritize the investigation of gene-environment interactions for MDD in humans. One interaction identified here is between genetic differences in *TERC* and childhood neglect which may predict MDD status in adulthood. Inflammatory cytokines show important interactions with the brain and may harbour the potential to be important biomarkers for MDD. Transcriptional differences in the chemokines *CCL24* and *CCR6* may act as useful biomarkers for MDD. Transcriptional differences in the *TNF* family and DNA methylation in *IL11* may act as useful predictors of antidepressant response. Furthermore, *ABCF1*, a gene coding for a translational regulator of the inflammatory cytokine pathway shows transcriptional changes in response to antidepressant treatment which correspond to antidepressant response, and so may represent an important novel drug target. If results reported here are replicated in larger studies in the future, this area of research may ultimately contribute towards the improved identification of individuals at risk for MDD, improved diagnosis of MDD, and may aid the development of targeted therapies for individual patients on the basis of their genetic background.

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Appendices

Appendix A: Powell et al., 2012 paper on depression-related behavioural tests in mice.

Depression-Related Behavioral Tests

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ABSTRACT

Overlapping characteristics between human depressive phenotypes and mouse behaviors has led to the creation of mouse models that aim to investigate the pathophysiology and treatment of unipolar depression. Behavioral tests in mice are used to assess and quantify the extent to which a mouse model displays a depression-like phenotype. The forced swim test and tail suspension test, sucrose preference test, and novelty suppressed feeding tests all aim to measure different components of depression. However, each one of these tests has different strengths and weaknesses in terms of predictive, face and construct validities. Furthermore, the responses to these tests vary greatly depending on strain of mouse. Depression-related behavioral tests are an extremely useful investigative tool in unearthing causes and predicting treatment outcomes in human depression, but as this review demonstrates, the comprehension of the finer details are extremely important in the design, analysis, and evaluation of such mouse studies. *Curr. Protoc. Mouse Biol.* 2:119-127

Keywords: depression • forced swim test • tail suspension test • novelty suppressed feeding • strain effect

INTRODUCTION

Depression is a mood disorder that consists of a distinct collection of phenomenological features including lowered mood, loss of interest or pleasure (anhedonia), and sleep and eating disturbances (DSM-IV; American Psychiatric Association, 1994). There are some features of depression that show homologies to mouse behaviors, and, as such, mouse models have been developed to improve our understanding of the pathophysiology and treatment of depressive symptoms. In particular, mouse models can be used to investigate the effects of stress on behavior (Meaney et al., 2001), test for novel antidepressants (Jacobson and Cryan, 2007), and investigate pharmacogenetic interactions (Malki et al., 2010).

There are numerous methods of modeling depression in mouse. For instance, the chronic mild stress model involves mice being subjected to successive administrations of a variety of mild stressors (Willner et al., 1992), whereas the maternal separation model involves separating a pup from its mother, to induce early stress in the developing mouse (Murgatroyd et al., 2009). In order to test whether these methods are successful in modeling features of depression, a range of behavioral tests have been developed to quantify and measure depressive-phenotypes. Behaviors such as immobility in the forced swim test and tail suspension tests are thought to mirror behavioral despair present in human depression (Porsolt et al., 2001). Anhedonia is modeled by a decrease in sucrose consumption in the sucrose preference test (Papp et al., 1991) and by an increase in latency to approach and eat food in the novelty-suppressed feeding test (Dulawa and Hena, 2005). Historically, a range of more severe and stressful tasks such as learned helplessness have been used to assess depression-like behaviors, but as many of these tests are now considered unethical (Cryan and Mombereau, 2004), these will not be discussed further. In the following overview, we elaborate on these four depression-related behavioral tests, discussing the validity of each test (see The Concept of Validity below), the influence of genetic background on the responses elicited by these tests, the use of automated methods of scoring, and the value of combining these tests in a test battery.

THE CONCEPT OF VALIDITY

Validity is usually subcategorized into three forms: face validity, construct validity, and predictive validity. Face validity refers to the

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phenomenological similarity between the behavior exhibited by the animal model and the specific symptoms of the human condition (Petit-Demouliere and Bourin, 2005), i.e., the degree to which a mouse behavior resembles the human behavior being modeled. Construct validity refers to the degree to which the test reflects the underlying theoretical assumptions (Geyer and Markou, 2002), i.e., are the biological mechanisms mediating the responses in a test the same as those underlying the human behavior being modeled?. Lastly, predictive validity is a term used to describe how accurately the test can predict the response found in humans based on the responses from the mouse model. For example, if a test in mice shows a novel drug has an antidepressant effect, does this effect translate to humans? Behavioral tests differ in their ability to satisfy each type of validity, and so conclusions made from depression-related behavioral tests should be viewed with these limitations in mind.

THE FORCED SWIM TEST (FST)

The most well-established behavioral test for measuring antidepressant response is the forced swim test (FST), also known as the Porsolt test. After carrying out learning experiments in rats, Porsolt et al. (1978) discovered that some rats failed to perform in water maze tasks and would simply float passively (referred to as immobility) rather than display directed swimming towards an escape platform. This immobility behavior was found to be reversed on administration of antidepressants (Porsolt et al., 1978). The same phenomenon was subsequently found in mice where immobility could be reduced by administration of antidepressants and electroshock therapy (Porsolt et al., 1978) and increased following stressful experiences (Weiss et al., 1981; Zebrowska-Lupina et al., 1990).

The FST has subsequently been refined to a 6-minute test in which the mouse is placed into a transparent cylinder filled with water to a depth of 10 cm (any shallower depths and the mouse may use their tail to help them to balance). The water may either be at room temperature or heated, as mice are particularly susceptible to hypothermia. There should be at least 15 cm above the upper surface of the water to prevent the mouse climbing out and escaping. Once the mouse is placed in the water, there should be a minimum pre-exposure/habituation period of 2 min prior to recording movement. Subsequently, immobility is measured and is defined as the absence of swimming with the exception of the minimal movement of one leg required to keep the head above water (Crawley, 2007). Immobility may be scored manually by a trained observer blind to any group allocation, or using an automated system (discussed later in this review). Additionally, other behaviors are often assessed as part of a modified forced swim test, which include swimming and climbing behaviors (see Cryan et al., 2005a for more details).

Immobility measures are quantified by latency to and frequency and duration of immobility, which may be represented as a percentage immobility score. Effective antidepressant therapies are known to decrease the time spent immobile. A study by Borsini and Meli (1988) found that 94% of all antidepressants were effective in reducing immobility and 83% of all classes of antidepressants were effective (see Table 1). Furthermore, it was found that the test discriminated against antipsychotic and anxiolytic medications (Borsini and Meli, 1988). As such, the forced swim is considered to have high predictive validity and has been adopted as a screening test for novel antidepressant drugs by the pharmaceutical industry.

The immobility in the FST has been proposed to model "behavioral despair," a behavior found in human depressed patients, as demonstrated by a shift in active coping to passivity in the test (Porsolt et al., 1978). However, the validity of this assumption and the FST has been heavily criticized. For example, a mouse swimming in a cylinder is considered a non-ethologically relevant stressor and thus it may be inappropriate to relate results from this test to human depressive symptoms. Furthermore, considering immobility in terms of a shift from active coping to passivity is anthropomorphic (Holmes, 2003). In fact, it has been suggested that energy conservation achieved by immobility may actually be a more efficient form of coping (West, 1990) and that immobility represents a switch from an active to a passive coping strategy. Therefore, FST may have some superficial face validity but low construct validity.

Holmes (2003) sums up the FST as a test that has "no direct, empirical relation to depression symptoms in humans, but which are nonetheless exquisitely sensitive to monoaminergic manipulations." This description largely reflects the received wisdom found in the current literature. However, researchers should be aware that although the FST is sensitive to monoaminergic drugs, it is not specific,

Table 1 A Summary of the Pharmacology of the Four Standard Mouse Tests of Depression

Behavioral test	Drug class	Example drug	Dose	Study
Forced swim	Tricyclic	Desipramine	4-16 mg/kg	David et al. (2004)
	SSRI	Citalopram	4-16 mg/kg	David et al. (2004)
	SNRI	Venlafaxine	10-40 mg/kg	Dhir and Kulkami (2007)
	Atypical	Bupropion	2 mg/kg and 4 mg/kg	David et al. (2004)
Tail suspension	Tricyclic	Imipramine	60-120 mg/kg	Cryan et al. (2005b)
	SSRI	Fluoxetine	30-120 mg/kg	Cryan et al. (2005b)
	SNRI	Venlafaxine	30-100 mg/kg	Cryan et al. (2005b)
	Atypical	Bupropion	8-16 mg/kg	Bourin et al. (2005)
Sucrose preference	Tricyclic	Imipramine	20 mg/kg	Monleon et al. (1995)
	SSRI	Fluoxetine	5 mg/kg	Muscat et al. (1992)
	MAOI	Moclobemide	20 mg/kg	Moreau et al. (1993)
	Atypical	Mianserin	5 mg/kg	Cheeta et al. (1994)
Novelty suppressed feeding	Tricyclic	Imipramine	20 mg/kg	Santarelli et al. (2003)
	SSRI	Fluoxetine	10-24 mg/kg	Dulawa et al. (2004)
	Atypical	Buspirone	4 mg/kg	Merali et al. (2003)

as manipulation of the dopamine (Yamada et al., 2004), histamine (Yanai et al., 1998; Perez-Garcia et al., 1999; Hirano et al., 2006), and acetylcholine (Mancinelli et al., 1988; Andreasen, 2008) systems have also been found to reduce immobility time in the FST. Furthermore, the FST seems to be less sensitive in its detection of selective serotonin reuptake inhibitors (SSRIs) and alterations to the serotonin system (Borsini, 1995; Binder et al., 2011). Sensitivity to SSRIs seems to increase in rats through the use of a two-trial FST in which immobility is measured on the second trial (e.g., Detke et al., 1995). However, it is currently unclear whether two trials of the FST in mice would increase the sensitivity of the test in its detection of SSRIs. For a more in depth review of the pharmacology of the forced swim test, see Petit-Demouliere and Bourin (2005).

Studies have revealed that different strains of mice respond differently to antidepressants in the FST and that genetic background exerts a strong influence in this test (Binder et al., 2011). Lucki et al. (2001) estimated that there was a ten-fold difference in baseline immobility scores across strains for the FST. As such, different strains have different baseline levels of behaviors, and the result of one strain in the FST should not be generalized to all strains of mice. O'Neil and Moore (2003) argued that responses to the FST were on a continuum with the DBA/2 mice on one extreme, showing very little immobility, and BALB/cJ mice on the other extreme, displaying >75% immobility. One strain of mice that is notoriously nonresponsive in the FST is the FVB strain (Jacobson and Cryan, 2007), which typically displays no immobility in the FST and, as such, cannot be used to evaluate antidepressant activity. Furthermore, antidepressant response in the FST is also dependent on genetic background. For instance, Lucki et al. (2001) compared responses to desipramine (NSRI) and fluoxetine (SSRI) in the FST across 11 strains of mice. Immobility decreased in 7/11 of the strains in response to desipramine but only 3/11 strains responded to fluoxetine.

TAIL SUSPENSION TEST (TST)

The tail suspension test is based on the observation that when a mouse is suspended by its tail from which it cannot escape, it will initially demonstrate escape-orientated behaviors, followed by an immobile posture; an effect that is reversed by administration of antidepressants (see Table 1). The typical duration of this test is 6 min and the latency to, and duration of, immobility is taken as the measure of behavioral despair. The TST is likened to a "dry" version of the FST (Porsolt et al., 2001), and as such, a greater time spent immobile represents increasing behavioral despair. Despite the conceptual similarity

between the TST and FST, there are subtle differences, which are reflected in opposing findings in the assessment of antidepressant drugs (Porsolt and Lenegre, 1992; Mombereau et al., 2004). For instance, Bai et al. (2001) found that the TST showed a dose-dependent decrease in immobility in the C57BL/6 mouse in response to imipramine, which was not found in the FST. Interestingly, one study utilized the subtle differences between the TST and FST in different strains of mice to develop a decision tree to determine the mechanism of action of a novel antidepressant drug. For instance, if a putative antidepressant responded positively in the FST in the Swiss mouse strain, and negatively in the TST in the NMRI mouse strain, the drug would likely be a noradrenaline reuptake inhibitor (Bourin et al., 2005). For an in-depth review of the differences between FST and TST, see Cryan et al. (2005b).

The TST suffers from the same limitations as the FST in face and construct validity. Nevertheless, the TST has good predictive validity and has value as a drug screening test. There have been numerous studies that have shown the effectiveness of antidepressants in reducing the time spent immobile in mice subjected to the TST (Steru et al., 1985; Perrault et al., 1992; Ripoll et al., 2003); see Table 1. Additionally, the TST is able to distinguish antidepressants from other types of drugs such as medications used to treat psychoses, anxiety and attention-deficit hyperactivity disorder (Cryan and Holmes, 2005).

Genetic background also influences behavioral responses to the TST (Lad et al., 2010). Liu and Gershenfeld (2001) examined the behavior of eleven strains of mice subjected to the TST both at baseline and after imipramine treatment. They found a significant difference in response to the TST across the eleven strains and found a heritability estimate for basal TST to be around 0.31, suggesting that genetic variation contributes moderately to the variance found in the TST. Furthermore, it was revealed that three strains in particular respond effectively to imipramine treatment in the TST (DBA/2J, NMRI and FVB/NJ), with the remaining eight strains failing to show changes in the TST. Similarly, Trullas et al. (1989) investigated the TST in drug-naïve mice across nine inbred strains and discovered that durations of immobility were shortest in DBA/2J and longest in BALB/cJ.

One strain of mouse in particular has been deemed inappropriate to use for the TST, which is the C57BL/6 inbred mouse strain, due to its ability to climb its own tail (Mayorga and Lucki, 2001); these mice, therefore, do not establish a typical immobile posture. However, the TST equipment can be modified to prevent tail climbing by using a cone placed around the tail (Lad et al., 2007).

SUCROSE PREFERENCE TEST (SPT)

Sucrose is used frequently in mouse studies as a reward due to its actions on the dopamine system (Cannon and Palmiter 2003). Most rodents when presented with sucrose solutions will choose to drink these over water due to their rewarding properties (Berridge, 2007). However, rodents subjected to stress show a general decrease in sensitivity to reward (anhedonic behavior), and, as such, show a decreased sucrose preference (Willner et al., 1987). For instance, male Alderley Park (AP) mice subjected to unpredictable chronic mild stress (UCMS) showed reduced consumption of 2% or 4% sucrose solutions (Monleon et al., 1995). Interestingly, the decrease in sucrose preference can be reversed on chronic administrations of antidepressants such as imipramine (Monleon et al., 1995); see Table 1 for a summary of drug responses in this test.

Whether or not sucrose preference is exactly parallel to the decreased response to reward processes found in human depressed patients is not clear, but regardless there is probably greater validity behind its construct than the more abstract immobility response to the TST and FST. Additionally, the SPT has much higher face validity, as there have been comparable tests done in depressed human patients with similar results (Wichers et al., 2009). Furthermore, the sucrose preference test only reveals changes in response to antidepressants after the mouse has been exposed to stress, a known risk factor involved in the causality of depression (Caspi et al., 2003). This mimics the ability of antidepressants to significantly improve symptom outcomes in depressed individuals who have experienced stressful life events (Keers et al., 2010).

Once again, genetic background can play an important role in determining the response to this behavioral test. One study revealed differences in UCMS sensitivity with decreased sucrose intake in CBA/H and C57BL/6 but not in DBA/2 strain of mouse (Pothion et al., 2004).

Interestingly, genetic contributions other than propensity to stress may account for variance between strains in the SPT. Lewis et al.

(2005) examined twelve strains of mice and discovered that intake of sucrose at low concentrations was correlated to variants of the Tas1r3 taste receptor gene, i.e., an independent factor other than stress. Moreover, Bachmanov et al. (1997) discovered two loci on chromosome 4 containing genes believed to be important in peripheral sensory sensitivity. These loci were found to account for over 50% of genetic variability in sucrose intake between C57BL/6ByJ and 129/J strains. Therefore, ability to taste sucrose is an important factor in the selection of the background strain of mouse to use in the SPT.

NOVELTY-SUPPRESSED FEEDING (NSF)

Novelty-suppressed feeding (NSF) or "hyponeophagia" tests assess anhedonia in a situation where there is a conflict between food reward and anxiety (Ansorge et al., 2004). NSF measures the latency of a mouse in approaching and eating food in a novel environment following an extended period (up to 24 hr) of food deprivation. The NSF is thought to capture the conflict a mouse faces in approaching and consuming a desirable food and avoiding exploration of the new environment (Dulawa and Hena, 2005). The NSF test usually involves a mouse being removed from its home cage and being placed in the corner of a novel test box, in which there is a single pellet of food (chow) placed in the center. The latency to approach the chow and begin eating is recorded within a 3-min period. If the mouse is anxious, it will avoid the food and display limited exploration of the test environment, whereas less anxious mice will approach the food quickly and begin eating (Crawley, 2007). It has been found that chronic mild stress can increase the latency for a mouse to approach chow in the NSF test—an effect that is reversed on administration of antidepressants (Surget et al., 2008); see Table 1.

Studies have demonstrated that chronic, as opposed to acute treatments with antidepressants are required to show a significant reduction in latency to NSF. Tricylic antidepressants (Bodnoff et al., 1988), SSRIs (Santarelli et al., 2003; Dulawa et al., 2004), and atypical antidepressants (Merali et al., 2003) are all required in chronic doses in order to elicit a reduction in latency. Thus, the NSF has some useful predictive value, as it reflects the therapeutic delay observed in the clinic (Skolnick, 1999). Furthermore, decreased latency responses to the NSF in response to antidepressants have been associated with changes in hippocampal neurogenesis—a process that is thought to be important in the recovery from depression in humans (Santarelli et al., 2003). Thus, the NSF displays useful construct validity.

A study by Trullas and Skolnick (1993) revealed considerable differences in baseline responses to NSF across seven inbred strains of mice. Strains were found to cluster into four groups ranging from "nonreactive" (strains that demonstrate low levels of latency), such as the BALB/cJ strain, to "highly reactive" (strains that demonstrate high levels of latency), such as the A/J strain. Consequently, this suggests that genetic background influences baseline levels of behavior in NSF and are likely to impact any assessment of response to stress and drugs, and this should be considered when designing studies using the NSF test.

TEST BATTERIES

Test batteries that combine well-established, robust tests of behavior allow us to extract latent traits that may better represent the underlying construct and be less affected by test-specific or laboratory-specific effects. In addition, test batteries can be an efficient way of obtaining the maximum amount of information per animal than would separate behavioral studies (Lad et al., 2010). Often, test batteries will consist of a set of behavioral tests that aim to measure different aspects of depression. However, behavioral tests themselves can often be considered stressful and may have unwanted additional effects on the mouse model. As stressful events are known to modulate behavior (Weiss et al., 1981; Zebrowska-Lupina et al., 1990), neurochemistry (Welch and Welch, 1968), and gene expression (Meaney, 2001) in mice, the order of tests in a battery is important. The general rule of thumb is to begin with the least stressful test and end with the most stressful test to minimize interference of one test on the outcome of subsequent tests (Lad et al., 2010). For instance, the SPT, followed by the NSF test, followed by the FST would seem an appropriate series of behavioral events. It is recommended that there should be at least a one day rest period between each of the tests in a behavioral battery (Lad et al., 2010). Strain-specific differences in terms of the duration of the rest period are largely unexplored, and thus it may be that some strains will require longer than others to recover. In addition, tasks that

measure general locomotor activity, anxiety, and overall physical fitness of the mouse can be incorporated in the battery to improve the sensitivity and robustness of any depression phenotypes assessed in the battery (Lad et al., 2010). Given the more severe nature of the depression tests (FST, TST, SPT, NSF), we recommend that only two or three tests be included in a test battery. In particular, the FST and TST are stressful procedures in their own right and researchers should be aware of the ethical implications of using such tests in combination, and/or selecting these tests to assess behavioral effects of another stressful procedure (Cryan and Holmes, 2005).

MANUAL VERSUS AUTOMATED MEASUREMENT OF FST AND TST

There are two methods of scoring immobility—manually or using an automated system. Manual scoring by a trained observer is both labor-intensive and subject to human error; therefore, systems were developed to automate scoring of the FST and TST (Crawley et al., 2007). Crowley et al. (2004) compared the use of the automated SMART II Video Tracking System software (San Diego Instruments) with manual expert ratings. There were significantly positive correlations between the automated tracking system and manual raters, with an average correlation of $r = 0.86$. Thus, such automated systems may be accurate enough to be used to quantify immobility in the FST. One of the main drawbacks of using an automated system is that other behaviors such as climbing and swimming are not usually measured, which may contain valuable information relating to the mechanism of action of novel drugs (Cryan et al., 2005a). However, Kurtuncu et al. (2005) used a different system that did have the ability to monitor swimming and climbing behaviors and found moderately high correlations between manual raters and the automated system.

Immobility in the TST can also be quantified using automated methods. Lad et al. (2007) used an automated tracking system and EthoVision (Noldus Netherlands) technology to quantify immobility in a modified two-trial TST. Automated ratings were compared to manual ratings and showed positive correlations of between $r = 0.58$ and $r = 0.78$ ($p < 0.001$). In contrast, Crowley et al. (2004) compared manual and automated ratings for immobility in the TST but using a strain gauge and a commercially available automated detection system (Med Associates). This study revealed an $r = 0.97$ average correlation between manual ratings and the automated ratings ($p < 0.001$), suggesting this automated method of assessing TST is particularly accurate.

SURVIVAL ANALYSIS OF LATENCY MEASURES

Latency measures of immobility in the FST and TST, and latency to feeding in the NSF test, are primary readouts for these tests. However, latency measures are not normally distributed, but rather they follow an exponential-like distribution (Fagan and Young 1978; Haccou and Meelis, 1992). A latency measure concerns the time to the occurrence of a single event, such as feeding in the NSF and, as such, latency measures typically have severely skewed distributions and are often censored (truncated) if an animal never displays the behavior to which latency relates, e.g., a mouse that constantly swims in the FST. Typically, behavioral researchers either assign an arbitrary score to censored latency values or exclude these values from their data sets so that they can use standard parametric statistics tests to analyze their data. Given the obvious limitations in such treatment of latency scores, we suggest that researchers should instead use a statistical method that can model the exponential-like distribution of the latency variable. Survival analysis, which partly originated from studying mortality in biological organisms (Collett, 2003), was specially designed for such analysis and deals appropriately with skewed distributions and censored data. One example of such a survival method of analysis that can be used for the analysis of behavioral data is Cox's Proportional Hazard Model (Lawless, 1982). The proportional hazard model is a general regression model, which makes no assumption regarding the type or shape of the underlying survival distribution, but assumes that the underlying hazard rate (rather than survival time) is a function of the independent variables.

CONCLUSION

This paper has emphasized the need to consider validity when interpreting depression-related behavioral tests. The FST, for instance, has high predictive validity, but low construct validity. Strain is a major factor influencing responses to behavioral tests both at baseline and in response to antidepressant drugs and, as such, generalizing results obtained from one strain of mouse to others should be avoided. Behavioral batteries are recommended as

they are cost-effective and they may be less influenced by test-specific or laboratory-specific biases. Automated methods are useful ways of scoring the FST and TST and correlate positively with manual ratings. Some methods, such as the strain gauge method in the TST, show particularly strong positive correlations with manual ratings. Analyzing results obtained from latency measures such as readouts from the FST, TST and NSF tests should be considered carefully and analyzed with statistical methods that deal correctly with censored data.

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Appendix B: Powell et al. in press. Article investigating the effects of mood-stabilizers on housekeeping gene expression stability.

Mood-stabilizers differentially affect housekeeping gene expression in human cells

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Key words: Reference genes, lithium, sodium valproate, gene expression normalization.

Abstract

Recent studies have revealed that antidepressants affect the expression of constitutively expressed "housekeeping genes" commonly used as normalizing reference genes in quantitative PCR (qPCR) experiments. There has yet to be an investigation however on the effects of mood-stabilizers on housekeeping gene stability. The current study utilized lymphoblastoid cell lines (LCLs) derived from patients with mood disorders to investigate the effects of a range of doses of lithium (0, 1, 2 and 5 mM) and sodium valproate (0, 0.06, 0.03 and 0.6 mM) on the stability of twelve housekeeping genes. RNA was extracted from LCLs and qPCR was used to generate cycle threshold (C_t) values which were input into RefFinder analyses. The study revealed drug-specific effects on housekeeping gene stability. The most stable housekeeping genes in LCLs treated: acutely with sodium valproate were *ACTB* and *RPL13A;* acutely with lithium were *GAPDH* and *ATP5B*; chronically with lithium were *ATP5B* and *CYC1.* The stability of *GAPDH* and *B2M* were particularly affected by duration of lithium treatment. The study adds to a growing literature that the selection of appropriate housekeeping genes is important for the accurate normalization of target gene expression in experiments investigating the molecular effects of mood disorder pharmacotherapies.

Introduction

Mood-stabilizers such as lithium and sodium valproate are commonly prescribed pharmacotherapies used in the treatment of bipolar disorder. Despite being clinically prescribed, the mechanisms of action of each of these drugs are still relatively unknown (Shaldubina et al., 2001). It is largely accepted however that gene expression changes associated with these drug treatments likely play a role in their therapeutic effects (Sugawara et al., 2010). Numerous studies using patient tissue samples such as brain and blood have attempted to unearth the molecular pharmacology of these mood-stabilizing drugs and how they confer their therapeutic effects (MacQuillin et al., 2007; Chetcuti et al., 2006; Tsuang et al., 2005). The quantitative polymerase chain reaction (qPCR) is the standard method of investigating candidate gene expression changes and is used to validate large-scale microarray expression hits (VanGuilder et al., 2008).

qPCR is a reaction in which a particular gene or region of DNA is amplified and detected in real-time. The relative quantification method of normalization is often employed for qPCR data and requires the subtraction of relatively stable reference genes' expression from the potentially more dynamic expression of the target gene (Livak & Schmittgen, 2001). As the reference genes used for normalization are subject to the same conditions as the target gene itself, it helps to control for variables such as RNA integrity and reverse transcription efficiency, as well as controlling for differences in the amount of starting material.

Most studies have used so-called 'housekeeping' genes as the reference genes for normalization, as the products of these genes are essential for basal cell metabolism and so are assumed to be expressed at a constant and detectable level in all nucleated cell types during all developmental stages (Thellin et al.,

1999). Many of the commonly used housekeeping genes such as beta-actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 18S rRNA (*18S*) and beta-2-microglobulin (*B2M*) were first used in traditional qualitative or semi-quantitative methods because of their high expression levels in all cells (Hendriks-Balk et al., 2007).

Recent research however suggests that housekeeping genes are not as stable as was originally thought, with evidence demonstrating different levels of stability in different cell types (Vandesompele et al., 2002) and conditions (Hruz er al., 2011). Previous studies have reported that antidepressants cause changes to the expression of housekeeping genes (Sugden et al, 2010; Powell et al., 2012). For instance, Sugden et al (2010) investigated the effects of antidepressants on housekeeping gene expression in a mouse fibroblast cell line. It was revealed that antidepressants affect housekeeping gene expression stability with some genes (ATP synthase and cytochrome c1) showing greater levels of stability than others [Eukaryotic translation initiation factor 4A2 (*Eif4a2*)].

Subsequently, the selection of appropriate housekeeping genes is important for the accurate normalization of gene expression experiments investigating the molecular effects of pharmacotherapies for unipolar depression. However, there is no evidence as to whether mood-stabilizers used to treat bipolar disorder also affect the expression of housekeeping genes. In this study we aimed to investigate the *in vitro* effects of the mood-stabilizers lithium and sodium valproate on the expression of a panel of candidate housekeeping genes using mood disorder patient-derived lymphoblastoid cell lines (LCLs) treated with these drugs. We aimed to establish: the most appropriate reference genes for LCLs treated with each drug, the most inappropriate reference genes for

LCLs treated with each drug, and the effects of drug treatment duration on housekeeping gene stability.

Materials and Methods

Participants

Samples in this study were collected as part of the Depression Case Control (DeCC) Study. The clinical methodology used in the DeCC collection was described in detail previously (see Gaysina et al., 2007). Briefly, subjects were identified from psychiatric clinics, hospitals, and general medical practices, and from volunteers responding to media advertisements. Only subjects of White European parentage were included. Subjects were over the age of 18 and had experienced two or more episodes of unipolar depression of at least moderate severity separated by at least two months of remission as defined by the Diagnostic and Statistical Manual 4th edition operational criteria (DSM-IV), or the International Classification of Diseases 10th edition operational criteria (ICD10). Participants were excluded if they had schizophrenia or bipolar disorder or their depression was caused by a physical illness, medication, alcohol or substance abuse, if they were intravenous drug users or were related to someone already recruited for the study. The study was approved by the Joint South London and Maudsley NHS Trust Institute of Psychiatry Research Ethics Committee and informed written consent was obtained from all the participants at the time of sample collection. 10 ml of whole blood from individuals was sent to the Human Genetic Cell Bank at the European Collection of Cell Cultures (ECACC), during which immortalised epstein-barr virus (EBV)-transformed lymphoblast cell lines (LCLs) were generated. The current study utilized LCLs

from five females diagnosed with unipolar depression, with a mean age of 53.6 ± 11.7 years, and who had not previously taken any mood-stabilizing medications.

Cell culture

The LCL samples were supplied by ECACC as frozen ampoules containing 1 mL of cells at a density of approximately 2 x 10^7 cells/mL, in cell culture freezing medium [10 % dimethyl sulphoxide (DMSO)]. Five cell lines were selected and grown in suspension in RPMI-1640 medium (Sigma-Aldrich, Poole, UK) supplemented with 10 % foetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin and 0.05 mg/mL neomycin at 37°C in a humidified atmosphere containing 5% CO_2 (see *S1*).

Drug Treatment of LCLs

The lithium chloride drug (Sigma-Aldrich) dilution was prepared by diluting the supplied 8 M LiCl solution in RNAase-free water to produce a sterile 1 M LiCl stock solution, and then further diluting the stock solution to 1, 2 and 5 mM for the treatment procedure. Sodium valproate (Sigma-Aldrich) was obtained in powder form and diluted in RNase-free water to a concentration of 3 mM. This stock solution was then diluted accordingly to concentrations of 0.06, 0.3 and 0.6 mM for the treatment procedure. A vehicle control dilution was also prepared using RNase-free water in serum-free supplemented growth medium.

Each of the five cell lines underwent an acute drug treatment as part of a four-stage culture protocol: (i) 72-hour growth phase, (ii) 24-hour serum-starve phase, (iii) 24-hour drug administration, and (iv) 24-hour recovery phase (for details see *S2*). After the final 24-hour recovery phase, cell pellets were obtained

following centrifugation and were immediately stored at -80 ºC for RNA extraction.

Each of the five cell lines were also treated with a chronic 7-day dose of LiCl. This involved the same doses as with the acutely treated cells but instead utilized a two stage culture protocol: (i) 72-hour growth phase and a (ii) 7-day lithium administration phase (see *S3* for details).

RNA extraction and cDNA synthesis

RNA extraction was performed using TRI reagent (Sigma-Aldrich). The purity and quantity of RNA was subsequently measured using the Nanodrop, ND1000, which showed all samples had 260/280 ratios of between 1.8 and 2.1. The RNA integrity numbers (RINs) of samples were assessed using the Agilent 2100 Bioanalyzer and all samples showed RINs > 9. The reverse transcription reaction was prepared using 1 μg of total RNA and the QuantiTect Reverse Transcription Kit (Qiagen, Crawley, UK). Complementary DNA (cDNA) synthesis was carried out in two steps: a genomic DNA (gDNA) wipe-out step, followed by reverse transcription step, as according to the manufacturer's protocol. Briefly, following gDNA removal, the samples were incubated for 15 minutes at 42°C with 1 uL Quantiscript Reverse Transcriptase, 5 X Quantiscript RT buffer, and 1 uL RT primer mix (oligo-dT and random primers). The reverse transcriptase enzyme was subsequently inactivated at 95°C for 3 minutes. The cDNA samples generated were stored at -20°C prior to use in the qPCR experiments.

Quantitative PCR

The qPCR experiments were performed in 384-well plates using reagents from Primer Design (Southampton, UK). Reagents included Precision-R MasterMix and the human geNorm Kit (PrimerDesign) which included 12 pre-designed PerfectProbe™ fluorescent (FAM-labelled) primer/probe sets for 12 human housekeeping genes. No C_t values for the housekeeping gene *Eif4a2* were detected in our samples. This was due to a technical problem with the primer/probe set that had been supplied for this gene. Therefore the gene *Eif4a2* was excluded from the assays and from the final analyses. Details of the remaining 11 housekeeping genes are shown in Table 1 (see *S4* for more detailed information on primers). The qPCR was set up according to the manufacturer's instructions, whereby each well on our 384-well plate contained a 20 µL qPCR reaction mixture consisting of: 5 µL cDNA, 4 µL RNase-free water, 1 µL of primer/probe mix and 10 µL of Precision-R MasterMix. qPCR assays were performed in duplicate to generate two technical replicates, and a negative control sample was also included for each reference gene.

The qPCR reactions were performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, California, USA). Thermal cycling conditions consisted of an enzyme activation stage (95°C for 10 minutes), followed by 50 cycles of a denaturation stage (95°C for 15 seconds) and hybridization and data collection stage (50°C for 30 seconds), and a final extension stage (72°C for 15 seconds). The software program SDS 2.1 (Applied Biosystems) generated C_t values (threshold cycle) from the data collected.

Accession No.	Gene Name	Protein function
NM 001101	Actin, beta (*ACTB*)	Cytoskeletal structural protein
NM 002046	Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	Glycolytic pathway enzyme
NM 021009	Ubiquitin C (*UBC*)	Protein modifier implicated in numerous functions
NM 004048	Beta-2-microglobulin (*B2M*)	Beta chain of major histocompatibility complex class I molecules
NM 003406	Tyrosine 3-monooxygenase /tryptophan 5-monoxygenase activation protein, zeta polypeptide (*YWHAZ*)	Adapter protein involved in mediating signal transduction
NM 012423	Ribosomal protein L13a (*RPL13A*)	Component of the 60S ribosomal subunit
NM 022551	18S ribosomal RNA (18S rRNA, *18S*)	Component of the 40S ribosomal subunit
NM 001916	Cytochrome c-1 (*CYC1*)	Heme-containing component of cytochrome b-c1 complex of the mitochondrial respiratory chain
NM 004168	Succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*)	Involved in complex II of the mitochondrial electron transport chain
NM 003286	Topoisomerase (DNA) I (*TOP1*)	Enzyme that controls and alters the topology of DNA during transcription
NM 001686	ATP synthase (*ATP5B*)	Subunit of mitochondrial ATP synthase, produces ATP from ADP using a protein gradient across the membrane

Table 1: A list of the 11 housekeeping genes included in the human geNorm kit, accession number and protein function.

Statistical Analysis

Average C_t values from our technical replicates were input into The RefFinder statistical analysis web-based tool (available from http://www.leonxie.com/referencegene.php). This tool was used for evaluating the stability of putative reference genes using an integrative weighted analysis which incorporates results from four already established analyses used for the selection of reference genes. These four already established statistical analyses for the selection of reference genes include: geNorm [as described in Vandesompele et al. (2002)], Normfinder [as described in Andersen et al. (2004)], Bestkeeper [as described in Pfaffl et al. (2004)] and the comparative ΔC_t method [as described in Silver et al. (2006)]. Based on the rankings from each method, RefFinder assigns an appropriate weight to an individual gene and calculates the geometric mean of their weights for the overall final ranking.

geNorm analysis was also considered separately as it can additionally be used to calculate the optimal number of reference genes required for normalization purposes. It achieves this by calculating standard deviations of log-transformed expression ratios for all housekeeping genes and then carrying out a pairwise comparison of the standard deviation of a particular gene with each of the remaining other housekeeping genes. One by one, the least stable reference genes are excluded based on high M values (which represent expression stability scores), leaving at least two remaining genes that correspond to the most stably expressed genes. According to Vandesompele et al. (2002), a combination of two or more reference genes producing a V (variation measure) of $V < 0.15$ is sufficient for optimal normalization.

Results

(i) Number of reference genes required for accurate normalization

The geNorm approach was used to establish the number of reference genes for accurate normalization. The analysis revealed that two reference genes were optimal, as three reference genes would not increase accuracy above that obtained using two reference genes. The variation between the mean of the two most stable genes compared with that of the three most stable was V = 0.05 for acute lithium treated cells, V = 0.04 for chronic lithium treated cells, and V = 0.04 for acute sodium valproate treated cells; all of which are well below the threshold of V<0.15 proposed by Vandesompele, et al. (2002). The pairwise variation was relatively stable across all the comparisons, none of which exceeded the threshold value of 0.15 (see *S5-S7*). Nevertheless, the selection of the most stable housekeeping genes will likely increase the chances of detecting target gene expression differences of smaller magnitudes.

(ii) Acute treatment (24 hours) of LCLs with sodium valproate

The C_t values of 11 housekeeping genes in LCLs treated with the different concentrations of sodium valproate are shown in Figure 1A. The lowest generated mean C_t value, and therefore the most highly expressed gene was *18S* (16.87 ± 0.19 S.D). The highest mean C_t value and therefore the lowest expressing gene was *SDHA* (30.11 ± 0.67 S.D). RefFinder analyses revealed that *ACTB* and *RPL13A* were the two most stable reference genes producing stability scores of 1.32 and 1.68 respectively. The two most unstable reference genes were *SDHA* and *CYC1* producing stability scores of 9.24 and 10.24 respectively, see Figure 1B. Results from each of the individual analyses contributing to RefFinder

results (geNorm, Normfinder, Bestkeeper and the comparative ΔC_t method) can be found in the *S8*.

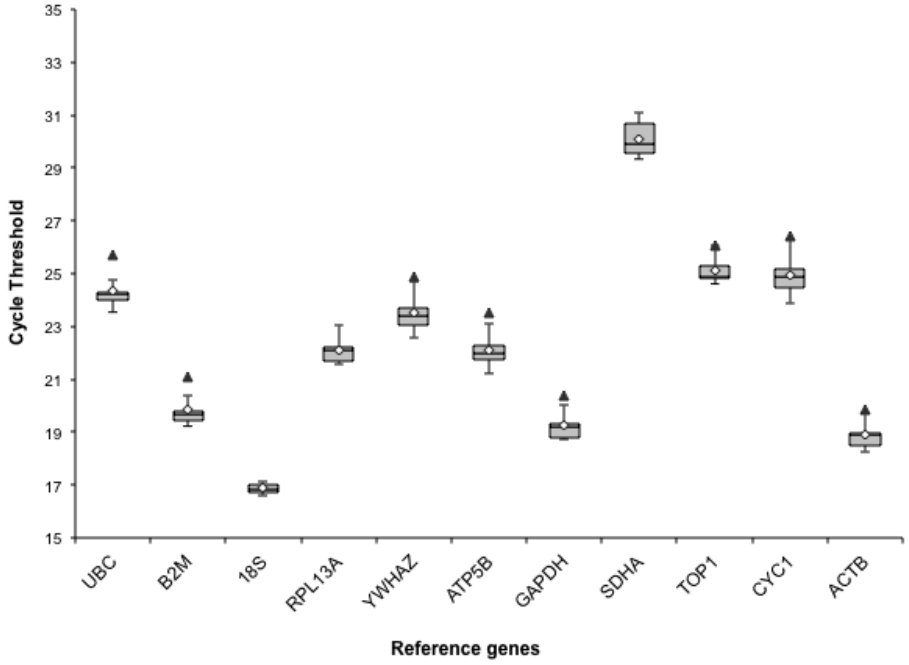

Figure 1A: qPCR cycle threshold values generated for 11 reference genes in LCLs treated with a range of sodium valproate concentrations (0, 0.06, 0.3 or 0.06 mM) for 24 hours. Expression levels are shown as median (lines), 25th percentile to the 75th percentile (boxes), and ranges (whiskers). The mean C_t values (white diamonds) and outliers (black triangles) are also indicated.

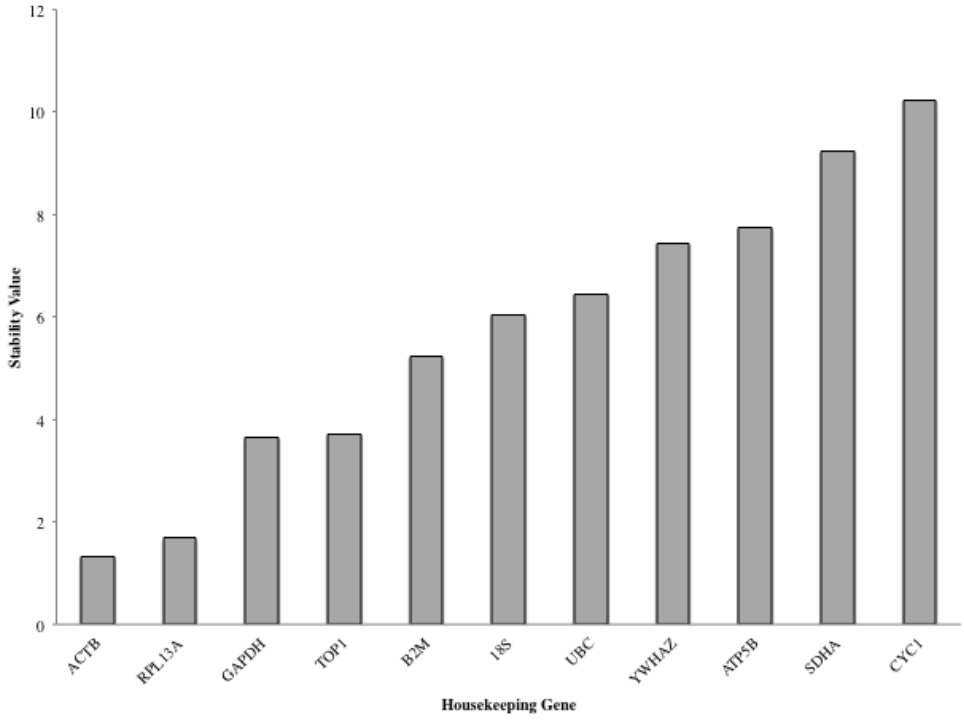

Figure 1B: Bar chart showing RefFinder expression stability values (y-axis) for 11 reference genes (x-axis) in LCLs treated with a range of sodium valproate concentrations (0, 0.06, 0.3 or 0.6 mM) for 24 hours.

(ii) Acute treatment (24 hours) of LCLs with lithium

The cycle threshold (C_t) values of 11 housekeeping genes in LCLs treated with different concentrations of lithium for 24 hours are shown in Figure 2A. The lowest mean C_t value, and therefore most highly expressed gene was *18S* (18.79 ± 1.74 S.D.), whilst the highest mean C_t value, and lowest expressed gene was succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*) (27.56 ± 1.45 S.D.). RefFinder analyses revealed that *GAPDH* and *ATP5B* were the two most stable reference genes producing stability scores of 2 and 2.63 respectively. The two most unstable reference genes were *SDHA* and *18S* producing stability scores of 9.49 and 11 respectively, see Figure 2B. Results from each of the

individual analyses contributing to RefFinder results (geNorm, Normfinder, Bestkeeper and the comparative ΔC_t method) can be found in the *S9*.

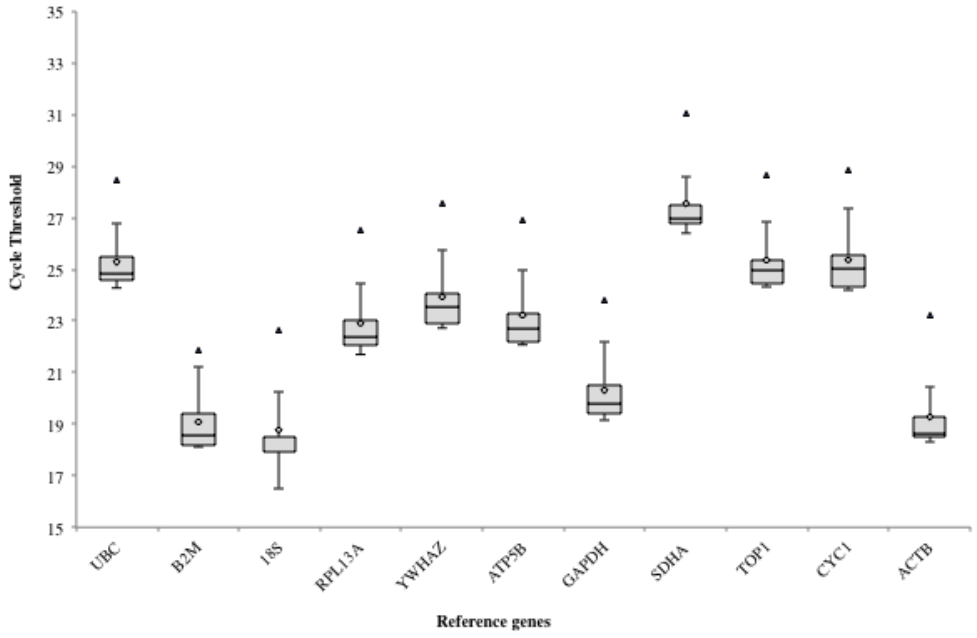

Figure 2A: qPCR cycle threshold values in 11 reference genes in LCLs treated with a range of lithium chloride concentrations (0, 1, 2 or 5 mM) for 24 hours. Expression levels are shown as median (lines), 25th percentile to the 75th percentile (boxes), and ranges (whiskers). The mean C_t values (white diamonds) and outliers (black triangles) are also indicated.

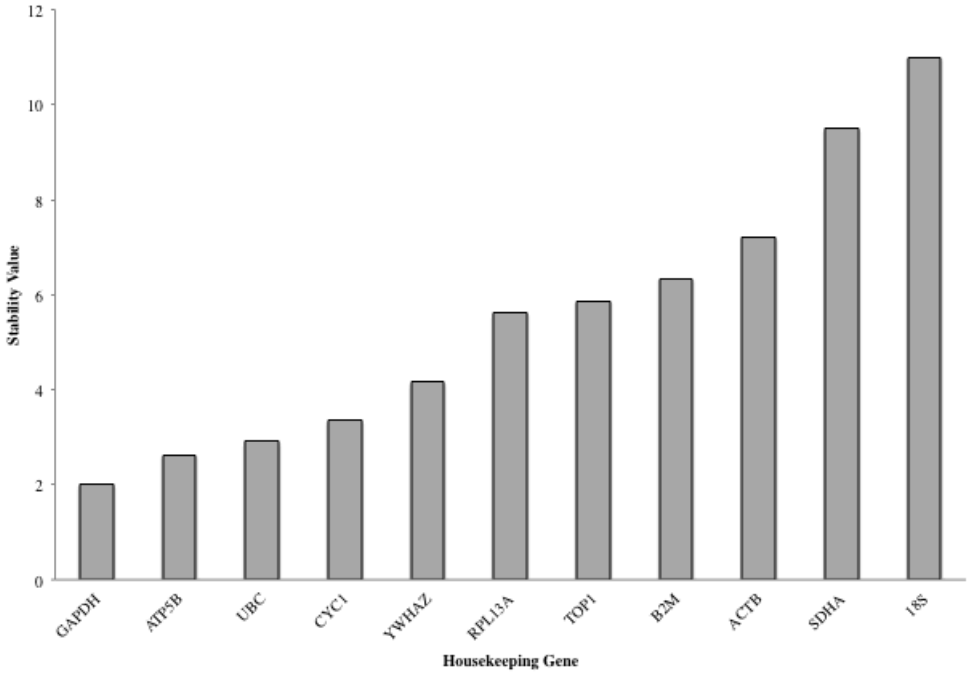

Figure 2B: Bar chart showing RefFinder expression stability values (y-axis) for 11 reference genes (x-axis) in LCLs treated with a range of lithium chloride concentrations (0, 1, 2 or 5 mM) for 24 hours.

(iii) *Chronic treatment (7 days) of LCLs with lithium*

The cycle threshold (C_t) values of 11 housekeeping genes in LCLs treated with different concentrations of lithium for 7 days are shown in Figure 3A. The lowest mean C_t value, and therefore most highly expressed gene was *18S* (18.26 ± 0.60 S.D.), whilst the highest mean C_t value, and lowest expressed gene was *SDHA* (29.28 ± 0.53 S.D.). RefFinder analyses revealed that *ATP5B* and *CYC1* were the two most stable reference genes producing stability scores of 1.78 and 2.63 respectively. The two most unstable reference genes were *SDHA* and *ACTB* producing stability scores of 10 and 11 respectively, see Figure 3B. Results from

each of the individual analyses contributing to RefFinder results (geNorm, Normfinder, Bestkeeper and the comparative ΔC_t method) can be found in the *S10*. A comparison of the stability values of the housekeeping genes in LCLs following acute and chronic lithium treatment are shown in Figure 4.

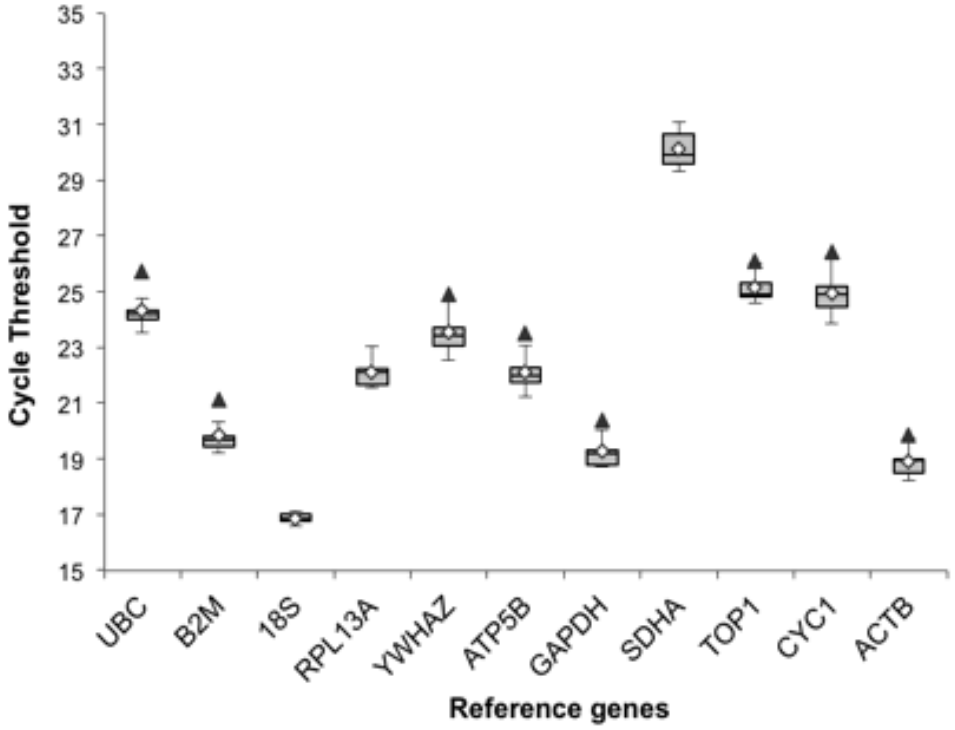

Figure 3A: qPCR cycle threshold values in 11 reference genes in LCLs treated with a range of lithium chloride concentrations (0, 1, 2 or 5 mM) for 7 days. Expression levels are shown as median (lines), 25th percentile to the 75th percentile (boxes), and ranges (whiskers). The mean C_t values (white diamonds) and outliers (black triangles) are also indicated.

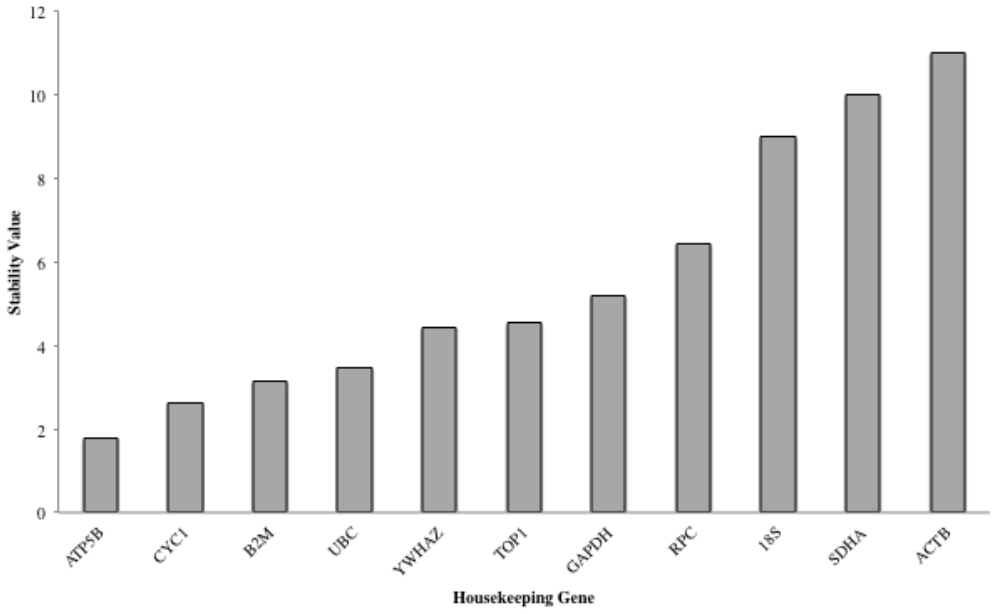

Figure 3B: Bar chart showing RefFinder expression stability values (y-axis) for 11 reference genes (x-axis) in LCLs treated with a range of lithium chloride concentrations (0, 1, 2 or 5 mM) for 7 days.

Discussion

Recent studies investigating the effects of antidepressants *in vitro* and *ex vivo* revealed that housekeeping gene expression, which was once believed to be 'stable' across conditions, in fact shows expression variability (Sugden et al., 2010; Powell et al., 2012). This in turn has consequences for studies investigating the gene expression effects of mood disorder pharmacotherapies using qPCR experiments and the relative quantification method of normalization.

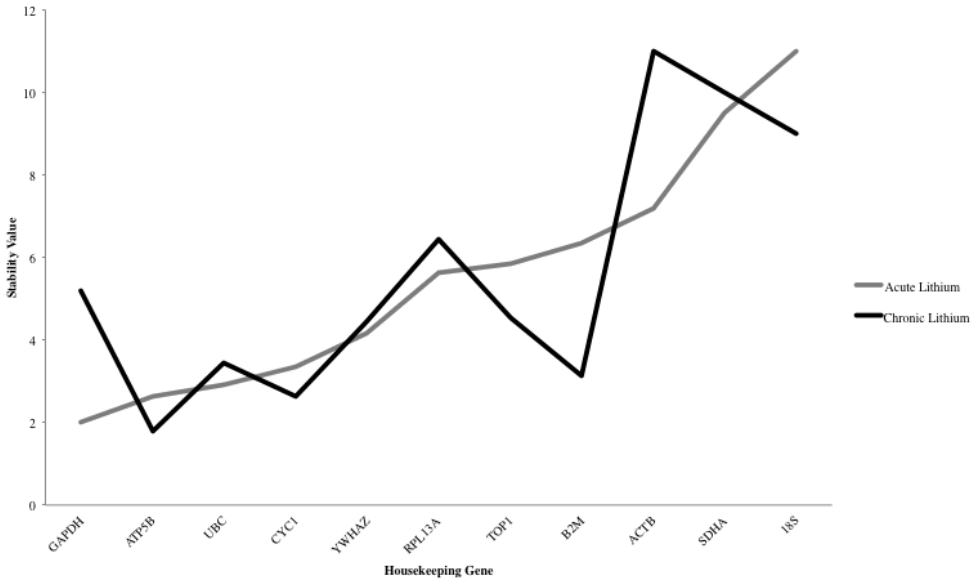

Figure 4: Comparison of the 11 reference gene expression profiles for LCLs treated with a range of lithium chloride concentrations (0, 1, 2 or 5 mM) for 24 hours (grey line) or for 7 days (black line). Gene names are shown on the x-axis and RefFinder stability values are marked on the y-axis.

The current study aimed to investigate the effects of the mood-stabilizers lithium and sodium valproate, which are used to treat bipolar disorder, on housekeeping gene expression. This study investigated the effects of acute drug treatments (sodium valproate, lithium) and a chronic drug treatment (lithium) at a variety of doses on the expression of a panel of eleven housekeeping genes. The drug dose ranges chosen incorporates doses that are believed to be therapeutic. This is based on serum concentrations of lithium found in bipolar disorder patients

(approximately 1 mM) (Taylor et al, 2007), and on the dose required for sodium valproate to demonstrate neuroprotective effects (approximately 0.1 mM) (Biermann et al., 2011). The current study used an *in vitro* experimental design carried out using LCLs derived from mood disorder patients. LCLs have previously been shown to have gene expression profiles which correlate highly with levels of gene expression in non-transformed lymphocytes and whole blood collected from psychiatric patients (Rollins et al., 2010). Consequently, these cell lines were specifically selected from mood disorder patients with the aim of creating the best *proxy* model for investigating the gene expression effects of mood-stabilizers that may occur *in vivo* in mood disorder patients.

The study revealed that all housekeeping genes were expressed at acceptably detectable levels under drug treatment conditions (i.e. Ct < 37) in LCLs producing a broad range of C_t values (see *Figures 1A*, *2A* and *3A*). Across all drug groups, *18S* was the most highly expressed housekeeping gene, whereas *SDHA* was the lowest expressing gene. Both mood-stabilizers were found to affect the stability of housekeeping gene expression and they did so differentially in a drug-specific manner. geNorm analyses revealed that two housekeeping genes were sufficient for optimal normalization of target genes in all drug treatment groups.

In the LCLs treated acutely with sodium valproate, the two most stable housekeeping genes according to the RefFinder analyses were *ACTB* and *RPL13A* (see *Figure 1B*). *ACTB,* a gene encoding a cytoskeletal structural protein, has previously been used as a normalizing reference gene in mice treated with sodium valproate (Wu et al., 2010) and has been shown to be one of the most stable housekeeping genes in studies investigating the effects of valproate on

forskolin-stimulated human adrenal carcinoma (H295R) cells (Krogh et al., 2010). *RPL13A,* a component of the 60S ribosomal subunit, has been shown to be one of the most stable housekeeping genes in a rat model of cerebral ischemia (Tian et al., 2007), but has not been previously used for normalization purposes in cells treated with sodium valproate. The housekeeping genes *SDHA* and *CYC1* showed the highest expression variability according to RefFinder analyses, and thus would be considered unsuitable as reference genes in LCLs exposed acutely to sodium valproate (see *Figure 1B*).

In the LCLs acutely treated with lithium, the two most stable housekeeping genes according to RefFinder were *GAPDH* and *ATP5B* (see *Figure 2B*). *GAPDH*, a gene encoding a glycolytic pathway enzyme has previously been shown to demonstrate stable gene expression in human epithelial cells treated with lithium (Nemeth et al., 2002) and has also previously been used for normalization in human placental cells treated with the drug (Roberts et al., 2007). *ATB5B*, a gene which encodes a protein involved in catalyzing ATP formation has previously been shown to be one of the most stable genes in cells treated with antidepressants (Sugden et al., 2010). *ATB5B,* was further shown to be the most stable reference gene in LCLs treated with lithium chronically along with *CYC1* (see *Figure 3B*). The greatest variation in expression and therefore worst reference genes in LCLs treated acutely with lithium were observed in the genes *SDHA* and *18S* (see *Figure 2B* and *Table 1*), and in *SDHA* and *ACTB* in LCLs treated chronically with lithium.

Previous research on the effects of antidepressants revealed that duration of drug treatment had little effect on housekeeping gene expression stability (Sugden et al., 2010). Here, we tested whether duration of treatment with a

mood-stabilizer might affect housekeeping gene expression. We observed the effects of a chronic (7 day) administration of lithium on housekeeping gene expression in LCLs, and compared it to an acute 24 hour treatment of lithium. The stability of genes in LCLs treated chronically showed a similar trend to the relative stability of genes in LCLs treated acutely. However, there were two genes which were key exceptions. In the chronically treated cells *GAPDH* became less stable and *B2M* became more stable (see *Figure 4*). Subsequently, *GAPDH* may be the most stable reference gene in LCLs treated acutely with lithium, but it is less suitable for LCLs treated chronically with the drug. If comparisons were to be drawn across different drug duration groups, *ATP5B* and *UBC* would in fact be considered two of the most stable reference genes (see *Figure 4*). These results consequently suggest that careful consideration is needed for the selection of reference genes not only for mood-stabilizer type but also for treatment duration.

To conclude, this is the first study to investigate the differential effects of mood-stabilizers on housekeeping gene expression in human cells from mood disorder patients. The study revealed that lithium and sodium valproate caused drug-specific effects on housekeeping gene expression stability, with lithium treatment duration also having some influences on housekeeping gene stability, particularly in the genes *GAPDH* and *B2M. ACTB* and *RPL13A* were the two most stable genes in LCLs treated acutely with sodium valproate, whereas *GAPDH* and *ATP5B* were the two most stable genes in LCLs treated acutely with lithium, and *ATB5B* and *CYC1* were the two most stable genes in LCLs treated chronically with lithium. *SDHA* was amongst the most variably expressed housekeeping genes across all drug groups and as such may be considered an

unsuitable reference gene in *in vitro* experiments investigating the effects of mood-stabilizers. The use of mood-disorder patient derived LCLs in this study arguably means results from this *in vitro* experiment might not only extrapolate to other *in vitro* studies investigating the effects of mood-stabilizers but also to *ex vivo* studies investigating gene expression in lymphocytes extracted from mood disorder patients; although this would need to be confirmed in future studies. The study provides further evidence to a growing literature that the selection of appropriate housekeeping genes is important for the accurate normalization of target gene expression in experiments investigating the molecular effects of mood disorder pharmacotherapies.

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Declaration of interest statement

Powell, Powell-Smith, Haddley, Quinn, Schalkwyk, and D'Souza report no competing interests. McGuffin and Farmer have received consultancy fees and honoraria for participating in expert panels from pharmaceutical companies, including Lundbeck and GlaxoSmithKline.

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Supplementary Information

S1: Cell culture method

The transformation of the B-lymphocyte component within the peripheral blood lymphocyte (PBL) population was performed through the culture of PBLs in a tissue culture medium infected with EBV supernatant, producing B lymphoblastoid cell lines. All cell lines were screened for mycoplasma contamination by PCR at ECACC. The human B lymphoblastoid cell lines were grown in suspension in 75 cm2 tissue culture flasks containing RPMI-1640 medium (Sigma catalogue number R0883) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin and 0.05 mg/mL neomycin at 37°C in a humidified atmosphere containing 5% CO2. All medium components were obtained from Sigma-Aldrich.

S2: Acute drug treatment of LCLs

The five LCLs were subcultured at 3 x 10^5 cells/mL and seeded into 6-well plates, using 2 mL of cells per well. In the initial stage, seeded samples were incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO2. In the 24-hour serum-starve phase: Following the growth phase, the samples were quantified and transferred to fresh 2 mL centrifuge tubes. The tubes were centrifuged for 2000 g for 5 minutes, following which the medium was aspirated to obtain cell pellets. The cell pellets were resuspended in 2 mL of fresh growth medium containing 0.1% Foetal Bovine Serum (FBS), reseeded in fresh 6-well plates, and incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO2. The next stage was the 24-hour lithium or sodium valproate administration: Each sample was then transferred to a fresh 2 mL centrifuge tube

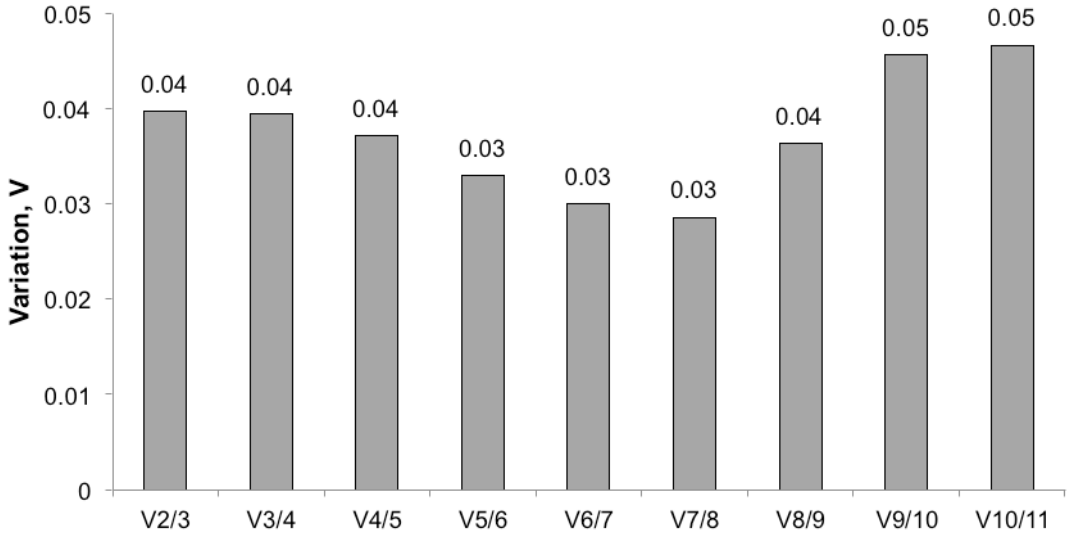
and cell pellets were obtained as above through centrifugation followed by aspiration of medium. The cells were resuspended in 2 mL of serum-free medium containing either a drug dilution or vehicle control reseeded in fresh 6-well plates and incubated for 24 hours as above. Lastly was the 24-hour recovery phase: Following the treatment phase, the samples were transferred to a fresh 2 mL centrifuge tube and cell pellets were obtained as above. The cells were resuspended in 2 mL of growth media containing full-serum (10%) FBS, then transferred to fresh 6-well plates and incubated for 24 hours as above. After the final 24 hour recovery phase, cell pellets were obtained as above, and these were immediately stored at -80°C for future RNA extraction.

S3: Chronic Lithium Treatment in LCLs

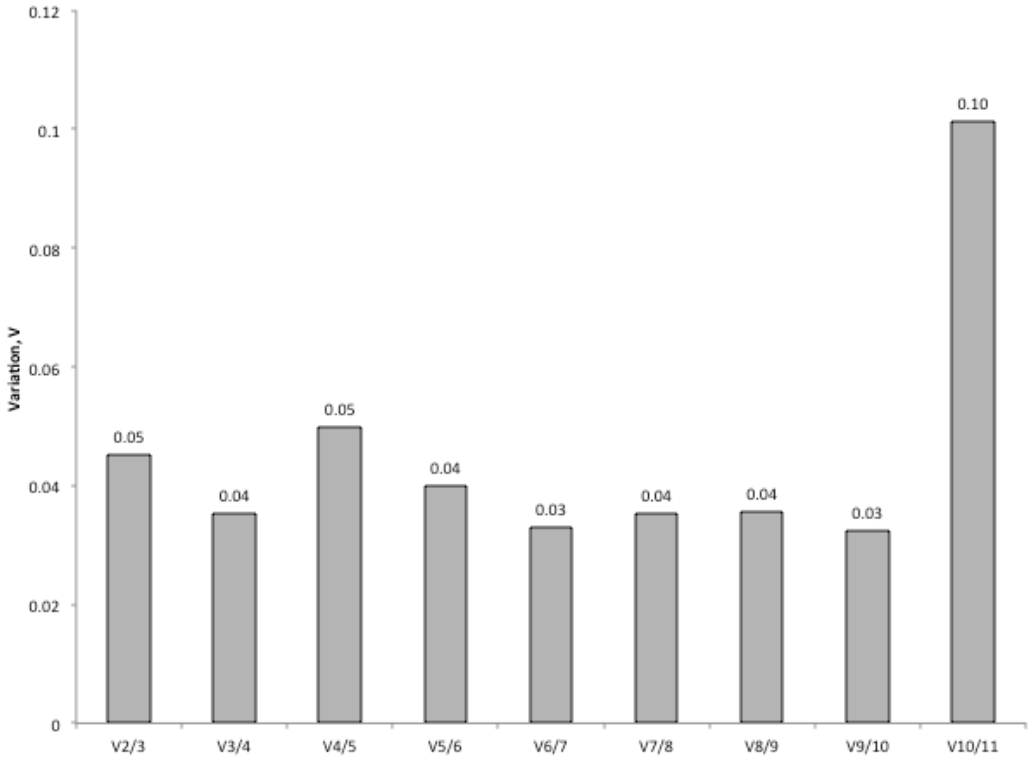
The samples were quantified and transferred to fresh 2 mL centrifuge tubes. The tubes were centrifuged and aspirated to obtain cell pellets, and the cells were resuspended in 2 mL of full-serum (10% FBS) growth medium containing either a drug or control dilution. The samples were then transferred to fresh 6-well plates, and incubated for 2 days as above. This was repeated twice more over the following 4 days. The samples were then transferred to fresh 2 mL centrifuge tubes and cell pellets were obtained as above. The cells were resuspended in 2 mL of serum-free growth medium containing either a drug or control dilution, reseeded in fresh 6-well plates, and incubated for a further 24 hours. Following the treatment phase, cell pellets were obtained as above and were immediately stored at -80°C for future RNA extraction.

Gene Name	Accession No.	Anchor Nucleotide	Context length sequence (bp)
ACTB	NM_001101	1195	106
GAPDH	NM_002046	1087	142
UBC	NM_021009	452	192
B2M	NM_004048	362	141
YWHAZ	NM_003406	2585	150
RPL13A	NM_012423	727	223
18S	M10098	235	99
CYC1	NM_001916	929	207
EIF4A2	NM_001967	900	146
SDHA	NM_004168	1032	154
TOP1	NM_003286	2361	195
ATP5B	NM_001686	1200	150

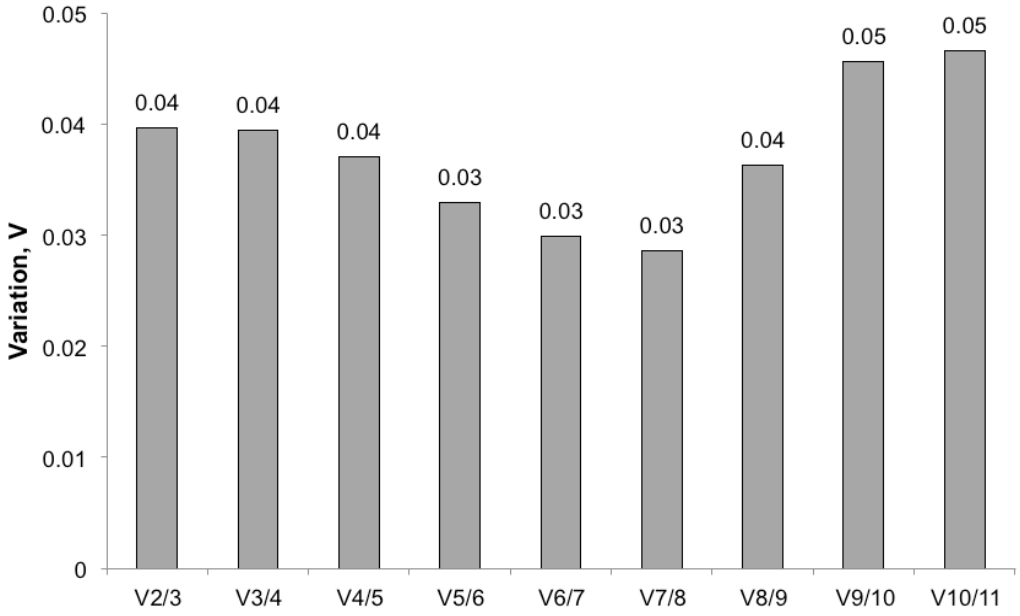
S4: Additional information on primers used, including accession number, anchor nucleotide and sequence length.



S5: Calculation of the optimum number of reference genes required for accurate normalization in LCLs treated with 0–0.6 mM sodium valproate for 24 hours. All comparisons of pairwise variations are below the recommended threshold of 0.15.



S6: Calculation of the optimum number of reference genes required for accurate normalization in LCLs treated with 0–5 mM lithium for 24 hours. All comparisons of pairwise variations are below the recommended threshold of 0.15.



S7: Calculation of the optimum number of reference genes required for accurate normalization in LCLs treated with 0–5 mM lithium for 7 days. All comparisons of pairwise variations are below the recommended threshold of 0.15.

Genes	geNorm	Normfinder	Bestkeeper	ΔC_t	RefFinder
ACTB	0.097	0.058	0.416	0.26	1.32
RPL13A	0.097	0.065	0.397	0.26	1.68
GAPDH	0.117	0.093	0.431	0.26	3.66
TOP1	0.143	0.078	0.43	0.27	3.72
B2M	0.166	0.164	0.492	0.29	5.23
18S	0.34	0.507	0.16	0.55	6.04
UBC	0.185	0.198	0.524	0.3	6.45
YWHAZ	0.201	0.206	0.532	0.32	7.45
ATP5B	0.217	0.231	0.506	0.33	7.74
SDHA	0.248	0.329	0.588	0.4	9.24
CYC1	0.295	0.468	0.596	0.51	10.24

S8: Relative gene expression stability values for each of the 11 housekeeping genes found in LCLs treated acutely with sodium valproate. Results are based on geNorm, Normfinder, Bestkeeper, the comparative ΔC_t method and RefFinder analyses.

Genes	geNorm	Normfinder	Bestkeeper	ΔC_t	RefFinder
GAPDH	0.153	0.024	0.988	0.33	2
ATP5B	0.128	0.165	1.037	0.34	2.63
UBC	0.223	0.154	0.874	0.37	2.91
CYC1	0.128	0.22	0.997	0.36	3.35
YWHAZ	0.143	0.181	0.988	0.36	4.16
RPL13A	0.197	0.193	1.055	0.36	5.62
TOP1	0.237	0.246	0.962	0.38	5.86
B2M	0.279	0.324	0.902	0.44	6.34
ACTB	0.257	0.213	1.017	0.42	7.2
SDHA	0.297	0.321	1.064	0.45	9.49
18S	0.448	1.111	1.214	1.13	11

S9: Relative gene expression stability values for each of the 11 housekeeping genes found in LCLs treated acutely with lithium. Results are based on geNorm, Normfinder, Bestkeeper, the comparative ΔC_t method and RefFinder analyses.

Genes	geNorm	Normfinder	Bestkeeper	ΔC_t	RefFinder
ATP5B	0.215	0.102	0.405	0.54	1.78
CYC1	0.252	0.096	0.403	0.55	2.63
B2M	0.221	0.277	0.314	0.59	3.13
UBC	0.232	0.246	0.398	0.59	3.46
YWHAZ	0.215	0.275	0.451	0.61	4.43
TOP1	0.278	0.248	0.383	0.6	4.53
GAPDH	0.29	0.184	0.427	0.59	5.18
RPC	0.244	0.276	0.427	0.6	6.44
18S	0.353	0.443	0.499	0.77	9
SDHA	0.533	1.249	0.705	1.37	10
ACTB	0.799	1.945	0.892	1.99	11

S10: Relative gene expression stability values for each of the 11 housekeeping genes found in LCLs treated chronically with lithium. Results are based on geNorm, Normfinder, Bestkeeper, the comparative ΔC_t method and RefFinder analyses.

Appendix C: ANOVA Results (Chapter 3)

Gene Family	Gene		Sum of Squares	df	Mean Square	F	Sig.	η_p^2
Complement	C3	Between Groups	3.557	2	1.779	1.188	0.311	0.032
		Within Groups	107.819	72	1.497			
		Total	111.376	74				
	C4A	Between Groups	3.441	2	1.721	1.008	0.369	0.023
		Within Groups	146.837	86	1.707			
		Total	150.278	88				
	C5	Between Groups	1.897	2	0.948	1.457	0.239	0.034
		Within Groups	54.67	84	0.651			
		Total	56.566	86				
Chemokine	CCL2	Between Groups	5.537	2	2.769	1.731	0.183	0.039
		Within Groups	137.524	86	1.599			
		Total	143.061	88				
	CCL3	Between Groups	0.267	2	0.133	0.215	0.807	0.005
		Within Groups	53.902	87	0.62			
		Total	54.168	89				
	CCL4	Between Groups	2.616	2	1.308	1.93	0.151	0.042
		Within Groups	58.961	87	0.678			
		Total	61.578	89				
	CCL5	Between Groups	0.488	2	0.244	0.309	0.735	0.007
		Within Groups	67.852	86	0.789			
		Total	68.34	88				
	CCL8	Between Groups	2.333	2	1.167	0.697	0.502	0.022
		Within Groups	105.472	63	1.674			
		Total	107.806	65				
	CCL16	Between Groups	3.178	2	1.589	1.533	0.222	0.036
		Within Groups	85.024	82	1.037			
		Total	88.202	84				
	CCL18	Between Groups	1.858	2	0.929	0.724	0.491	0.036
		Within Groups	50.038	39	1.283			
		Total	51.896	41				
	CCL19	Between Groups	29.083	2	14.542	1.36	0.264	0.041
		Within Groups	673.417	63	10.689			
		Total	702.5	65				
	CCL20	Between Groups	11.932	2	5.966	1.011	0.369	0.027
		Within Groups	436.873	74	5.904			
		Total	448.805	76				
	CCL23	Between Groups	6.46	2	3.23	1.786	0.174	0.040
		Within Groups	153.678	85	1.808			
		Total	160.138	87				
	CCL24	Between Groups	10.613	2	5.306	6.586	**0.002**	0.134
		Within Groups	68.487	85	0.806			
		Total	79.1	87				
	CCL25	Between Groups	11.433	2	5.716	1.248	0.292	0.029
		Within Groups	389.445	85	4.582			
		Total	400.878	87				
	CCL26	Between Groups	10.465	2	5.233	0.645	0.53	0.031
		Within Groups	324.311	40	8.108			
		Total	334.776	42				
	CXCL1	Between Groups	8.18	2	4.09	3.727	**0.028**	0.079
		Within Groups	95.478	87	1.097			
		Total	103.658	89				
	CXCL2	Between Groups	0.622	2	0.311	0.084	0.919	0.002
		Within Groups	295.105	80	3.689			
		Total	295.727	82				
	CXCL3	Between Groups	2.899	2	1.45	1.142	0.325	0.032
		Within Groups	86.327	68	1.27			
		Total	89.226	70				
	CXCL5	Between Groups	1.818	2	0.909	0.552	0.578	0.013
		Within Groups	143.337	87	1.648			
		Total	145.155	89				
	CXCL6	Between Groups	15.262	2	7.631	4.378	**0.015**	0.091
		Within Groups	151.652	87	1.743			
		Total	166.914	89				
	CXCL9	Between Groups	14.887	2	7.444	5.093	**0.008**	0.107
		Within Groups	124.219	85	1.461			
		Total	139.107	87				
	CXCL10	Between Groups	20.919	2	10.46	4.51	**0.014**	0.095
		Within Groups	199.454	86	2.319			
		Total	220.374	88				
	CXCL11	Between Groups	1.485	2	0.743	0.407	0.667	0.015
		Within Groups	96.611	53	1.823			
		Total	98.096	55				
	CXCL12	Between Groups	0.363	2	0.182	0.185	0.832	0.010
		Within Groups	34.364	35	0.982			
		Total	34.728	37				
	CXCL13	Between Groups	0.387	2	0.194	0.157	0.855	0.005
		Within Groups	85.118	69	1.234			
		Total	85.506	71				

Gene Family	Gene		Sum of Squares	df	Mean Square	F	Sig.	η_p^2
Chemokine Receptor	CCR1	Between Groups	3.02	2	1.51	1.988	0.143	0.044
		Within Groups	66.064	87	0.759			
		Total	69.084	89				
	CCR2	Between Groups	3.193	2	1.597	1.094	0.339	0.025
		Within Groups	126.935	87	1.459			
		Total	130.128	89				
	CCR3	Between Groups	1.982	2	0.991	0.502	0.607	0.011
		Within Groups	171.702	87	1.974			
		Total	173.684	89				
	CCR4	Between Groups	6.477	2	3.238	3.28	**0.042**	0.070
		Within Groups	85.886	87	0.987			
		Total	92.363	89				
	CCR5	Between Groups	0.472	2	0.236	0.225	0.799	0.005
		Within Groups	91.486	87	1.052			
		Total	91.958	89				
	CCR6	Between Groups	4.438	2	2.219	3.548	**0.033**	0.075
		Within Groups	54.405	87	0.625			
		Total	58.843	89				
	CCR7	Between Groups	4.678	2	2.339	2.759	0.069	0.060
		Within Groups	73.752	87	0.848			
		Total	78.43	89				
	CCR8	Between Groups	3.23	2	1.615	0.569	0.568	0.013
		Within Groups	247.13	87	2.841			
		Total	250.36	89				
	CCR9	Between Groups	6.269	2	3.135	3.334	**0.04**	0.071
		Within Groups	81.802	87	0.94			
		Total	88.071	89				
	CX3CR1	Between Groups	1.501	2	0.751	0.489	0.615	0.011
		Within Groups	133.396	87	1.533			
		Total	134.897	89				
	XCR1	Between Groups	10.489	2	5.245	6.332	**0.003**	0.127
		Within Groups	72.061	87	0.828			
		Total	82.55	89				
Interleukin	IL1A	Between Groups	2.967	2	1.484	0.579	0.563	0.017
		Within Groups	176.663	69	2.56			
		Total	179.63	71				
	IL1B	Between Groups	3.541	2	1.771	1.775	0.176	0.039
		Within Groups	86.781	87	0.997			
		Total	90.322	89				
	IL5	Between Groups	2.456	2	1.228	0.588	0.558	0.013
		Within Groups	179.571	86	2.088			
		Total	182.027	88				
	IL6	Between Groups	5.592	2	2.796	1.98	0.145	0.046
		Within Groups	117.242	83	1.413			
		Total	122.834	85				
	IL8	Between Groups	20.326	2	10.163	6.872	**0.002**	0.136
		Within Groups	128.665	87	1.479			
		Total	148.991	89				
	IL11	Between Groups	3.197	2	1.598	0.781	0.464	0.031
		Within Groups	100.336	49	2.048			
		Total	103.533	51				
	IL10	Between Groups	4.081	2	2.04	1.593	0.209	0.035
		Within Groups	111.444	87	1.281			
		Total	115.524	89				
	IL13	Between Groups	12.508	2	6.254	1.545	0.222	0.052
		Within Groups	226.643	56	4.047			
		Total	239.151	58				
	IL17C	Between Groups	0.536	2	0.268	0.274	0.761	0.006
		Within Groups	85	87	0.977			
		Total	85.536	89				
	IL22	Between Groups	2.815	2	1.408	1.064	0.351	0.031
		Within Groups	88.624	67	1.323			
		Total	91.439	69				
	IL36A	Between Groups	0.004	2	0.002	0.002	0.998	0.000
		Within Groups	86.904	77	1.129			
		Total	86.908	79				
	IL37	Between Groups	4.43	2	2.215	1.136	0.326	0.027
		Within Groups	159.819	82	1.949			
		Total	164.249	84				

Gene Family	Gene		Sum of Squares	df	Mean Square	F	Sig.	η_p^2
Interleukin receptor	IL1R1	Between Groups	0.209	2	0.105	0.082	0.921	0.002
		Within Groups	111.086	87	1.277			
		Total	111.295	89				
	IL1RN	Between Groups	1.044	2	0.522	0.662	0.518	0.015
		Within Groups	68.538	87	0.788			
		Total	69.582	89				
	IL5RA	Between Groups	4.096	2	2.048	1.863	0.161	0.041
		Within Groups	95.653	87	1.099			
		Total	99.749	89				
	IL8RA	Between Groups	1.705	2	0.852	1.039	0.358	0.023
		Within Groups	71.414	87	0.821			
		Total	73.119	89				
	IL8RB	Between Groups	2.343	2	1.171	1.16	0.318	0.026
		Within Groups	87.86	87	1.01			
		Total	90.203	89				
	IL9R	Between Groups	2.956	2	1.478	0.989	0.381	0.047
		Within Groups	59.756	40	1.494			
		Total	62.712	42				
	IL10RA	Between Groups	1.026	2	0.513	1.066	0.349	0.024
		Within Groups	41.861	87	0.481			
		Total	42.887	89				
	IL10RB	Between Groups	0.562	2	0.281	0.33	0.72	0.008
		Within Groups	74.094	87	0.852			
		Total	74.656	89				
	IL13RA1	Between Groups	0.824	2	0.412	0.571	0.567	0.013
		Within Groups	62.808	87	0.722			
		Total	63.632	89				
Tumor Necrosis Factor	TNF	Between Groups	0.701	2	0.35	1.036	0.359	0.023
		Within Groups	29.425	87	0.338			
		Total	30.126	89				
	LTA	Between Groups	1.309	2	0.655	1.036	0.359	0.023
		Within Groups	54.952	87	0.632			
		Total	56.261	89				
	LTB	Between Groups	1.108	2	0.554	0.824	0.442	0.019
		Within Groups	58.495	87	0.672			
		Total	59.604	89				
	CD40LG	Between Groups	0.321	2	0.161	0.425	0.655	0.010
		Within Groups	32.926	87	0.378			
		Total	33.247	89				
	LTB4R	Between Groups	4.044	2	2.022	2.249	0.112	0.049
		Within Groups	78.201	87	0.899			
		Total	82.244	89				
Other	BCL6	Between Groups	2.034	2	1.017	1.064	0.35	0.024
		Within Groups	82.208	86	0.956			
		Total	84.243	88				
	AIMP1	Between Groups	1.735	2	0.868	0.942	0.394	0.021
		Within Groups	80.118	87	0.921			
		Total	81.853	89				
	CEBPB	Between Groups	2.111	2	1.055	0.865	0.424	0.020
		Within Groups	106.101	87	1.22			
		Total	108.212	89				
	MIF	Between Groups	1.863	2	0.931	1.387	0.255	0.031
		Within Groups	58.406	87	0.671			
		Total	60.269	89				
	NR3C1	Between Groups	4.622	2	2.311	3.465	**0.036**	0.074
		Within Groups	58.029	87	0.667			
		Total	62.651	89				
	SPP1	Between Groups	13.166	2	6.583	1.937	0.15	0.043
		Within Groups	292.239	86	3.398			
		Total	305.404	88				
	TOLLIP	Between Groups	0.346	2	0.173	0.54	0.584	0.012
		Within Groups	27.869	87	0.32			
		Total	28.215	89				

Appendix B: Games-Howell Results (Chapter 3)

Gene Family	Gene	Subject Group	Comparison Group	Mean Difference	S.E.	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
Complement	C3	Control	MDD	-0.529	0.384	0.360	-1.458	0.400
			BPD	-0.419	0.299	0.351	-1.148	0.311
		MDD	Control	0.529	0.384	0.360	-0.400	1.458
			BPD	0.110	0.344	0.945	-0.725	0.945
		BPD	Control	0.419	0.299	0.351	-0.311	1.148
			MDD	-0.110	0.344	0.945	-0.945	0.725
	C4A	Control	MDD	-0.475	0.355	0.380	-1.330	0.379
			BPD	-0.316	0.340	0.624	-1.135	0.503
		MDD	Control	0.475	0.355	0.380	-0.379	1.330
			BPD	0.159	0.324	0.876	-0.621	0.939
		BPD	Control	0.316	0.340	0.624	-0.503	1.135
			MDD	-0.159	0.324	0.876	-0.939	0.621
	C5	Control	MDD	0.007	0.224	0.999	-0.532	0.546
			BPD	-0.312	0.200	0.272	-0.795	0.170
		MDD	Control	-0.007	0.224	0.999	-0.546	0.532
			BPD	-0.319	0.207	0.279	-0.818	0.179
		BPD	Control	0.312	0.200	0.272	-0.170	0.795
			MDD	0.319	0.207	0.279	-0.179	0.818
	CCL2	Control	MDD	-0.449	0.292	0.283	-1.154	0.256
			BPD	-0.588	0.363	0.247	-1.462	0.287
		MDD	Control	0.449	0.292	0.283	-0.256	1.154
			BPD	-0.139	0.326	0.905	-0.927	0.649
		BPD	Control	0.588	0.363	0.247	-0.287	1.462
			MDD	0.139	0.326	0.905	-0.649	0.927
	CCL3	Control	MDD	-0.112	0.209	0.855	-0.615	0.392
			BPD	-0.119	0.191	0.809	-0.579	0.342
		MDD	Control	0.112	0.209	0.855	-0.392	0.615
			BPD	-0.007	0.209	0.999	-0.509	0.495
		BPD	Control	0.119	0.191	0.809	-0.342	0.579
			MDD	0.007	0.209	0.999	-0.495	0.509
	CCL4	Control	MDD	-0.166	0.211	0.712	-0.674	0.341
			BPD	-0.415	0.223	0.159	-0.951	0.121
		MDD	Control	0.166	0.211	0.712	-0.341	0.674
			BPD	-0.249	0.204	0.445	-0.739	0.241
		BPD	Control	0.415	0.223	0.159	-0.121	0.951
			MDD	0.249	0.204	0.445	-0.241	0.739
	CCL5	Control	MDD	0.033	0.244	0.990	-0.554	0.621
			BPD	-0.137	0.232	0.825	-0.695	0.421
		MDD	Control	-0.033	0.244	0.990	-0.621	0.554
			BPD	-0.170	0.217	0.714	-0.693	0.352
		BPD	Control	0.137	0.232	0.825	-0.421	0.695
			MDD	0.170	0.217	0.714	-0.352	0.693
	CCL8	Control	MDD	-0.363	0.412	0.655	-1.372	0.646
			BPD	0.073	0.337	0.975	-0.747	0.892
		MDD	Control	0.363	0.412	0.655	-0.646	1.372
			BPD	0.436	0.431	0.575	-0.617	1.489
		BPD	Control	-0.073	0.337	0.975	-0.892	0.747
			MDD	-0.436	0.431	0.575	-1.489	0.617
	CCL16	Control	MDD	-0.291	0.266	0.522	-0.934	0.351
			BPD	-0.475	0.288	0.235	-1.170	0.220
		MDD	Control	0.291	0.266	0.522	-0.351	0.934
			BPD	-0.183	0.259	0.760	-0.809	0.443
		BPD	Control	0.475	0.288	0.235	-0.220	1.170
			MDD	0.183	0.259	0.760	-0.443	0.809

Gene Family	Gene	Subject Group	Comparison Group	Mean Difference	S.E.	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
	CCL18	Control	MDD	0.206	0.419	0.877	-0.903	1.314
			BPD	0.474	0.403	0.478	-0.532	1.481
		MDD	Control	-0.206	0.419	0.877	-1.314	0.903
			BPD	0.269	0.508	0.858	-1.016	1.553
		BPD	Control	-0.474	0.403	0.478	-1.481	0.532
			MDD	-0.269	0.508	0.858	-1.553	1.016
	CCL19	Control	MDD	1.544	1.095	0.351	-1.181	4.268
			BPD	0.503	0.324	0.279	-0.287	1.293
		MDD	Control	-1.544	1.095	0.351	-4.268	1.181
			BPD	-1.041	1.085	0.609	-3.745	1.664
		BPD	Control	-0.503	0.324	0.279	-1.293	0.287
			MDD	1.041	1.085	0.609	-1.664	3.745
	CCL20	Control	MDD	0.645	0.760	0.676	-1.230	2.520
			BPD	-0.295	0.316	0.622	-1.060	0.470
		MDD	Control	-0.645	0.760	0.676	-2.520	1.230
			BPD	-0.940	0.774	0.453	-2.842	0.962
		BPD	Control	0.295	0.316	0.622	-0.470	1.060
			MDD	0.940	0.774	0.453	-0.962	2.842
	CCL23	Control	MDD	-0.235	0.360	0.792	-1.102	0.632
			BPD	0.419	0.344	0.447	-0.409	1.248
		MDD	Control	0.235	0.360	0.792	-0.632	1.102
			BPD	0.654	0.348	0.153	-0.182	1.491
		BPD	Control	-0.419	0.344	0.447	-1.248	0.409
			MDD	-0.654	0.348	0.153	-1.491	0.182
	CCL24	Control	MDD	0.779	0.259	0.011	0.153	1.404
			BPD	0.102	0.181	0.839	-0.334	0.538
		MDD	Control	-0.779	0.259	0.011	-1.404	-0.153
			BPD	-0.676	0.249	0.025	-1.280	-0.072
		BPD	Control	-0.102	0.181	0.839	-0.538	0.334
			MDD	0.676	0.249	0.025	0.072	1.280
	CCL25	Control	MDD	0.130	0.537	0.968	-1.162	1.423
			BPD	-0.692	0.560	0.438	-2.042	0.658
		MDD	Control	-0.130	0.537	0.968	-1.423	1.162
			BPD	-0.822	0.579	0.337	-2.215	0.570
		BPD	Control	0.692	0.560	0.438	-0.658	2.042
			MDD	0.822	0.579	0.337	-0.570	2.215
	CCL26	Control	MDD	-0.992	1.481	0.785	-4.945	2.961
			BPD	-1.272	1.476	0.674	-5.220	2.676
		MDD	Control	0.992	1.481	0.785	-2.961	4.945
			BPD	-0.280	0.406	0.772	-1.292	0.732
		BPD	Control	1.272	1.476	0.674	-2.676	5.220
			MDD	0.280	0.406	0.772	-0.732	1.292
	CXCL1	Control	MDD	-0.738	0.261	0.017	-1.366	-0.111
			BPD	-0.385	0.274	0.345	-1.045	0.274
		MDD	Control	0.738	0.261	0.017	0.111	1.366
			BPD	0.353	0.276	0.413	-0.311	1.017
		BPD	Control	0.385	0.274	0.345	-0.274	1.045
			MDD	-0.353	0.276	0.413	-1.017	0.311
	CXCL2	Control	MDD	-0.202	0.586	0.937	-1.642	1.237
			BPD	-0.150	0.329	0.892	-0.945	0.645
		MDD	Control	0.202	0.586	0.937	-1.237	1.642
			BPD	0.053	0.614	0.996	-1.447	1.552
		BPD	Control	0.150	0.329	0.892	-0.645	0.945
			MDD	-0.053	0.614	0.996	-1.552	1.447

Gene Family	Gene	Subject Group	Comparison Group	Mean Difference	S.E.	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
	CXCL3	Control	MDD	0.270	0.322	0.680	-0.509	1.049
			BPD	-0.250	0.328	0.727	-1.042	0.542
		MDD	Control	-0.270	0.322	0.680	-1.049	0.509
			BPD	-0.520	0.314	0.233	-1.284	0.243
		BPD	Control	0.250	0.328	0.727	-0.542	1.042
			MDD	0.520	0.314	0.233	-0.243	1.284
	CXCL5	Control	MDD	-0.227	0.327	0.768	-1.013	0.560
			BPD	0.116	0.336	0.937	-0.692	0.923
		MDD	Control	0.227	0.327	0.768	-0.560	1.013
			BPD	0.342	0.331	0.560	-0.455	1.139
		BPD	Control	-0.116	0.336	0.937	-0.923	0.692
			MDD	-0.342	0.331	0.560	-1.139	0.455
	CXCL6	Control	MDD	-1.007	0.347	0.015	-1.848	-0.167
			BPD	-0.459	0.294	0.270	-1.166	0.248
		MDD	Control	1.007	0.347	0.015	0.167	1.848
			BPD	0.549	0.377	0.319	-0.358	1.456
		BPD	Control	0.459	0.294	0.270	-0.248	1.166
			MDD	-0.549	0.377	0.319	-1.456	0.358
	CXCL9	Control	MDD	-0.517	0.347	0.303	-1.352	0.318
			BPD	0.487	0.274	0.187	-0.174	1.149
		MDD	Control	0.517	0.347	0.303	-0.318	1.352
			BPD	1.004	0.317	0.007	0.239	1.770
		BPD	Control	-0.487	0.274	0.187	-1.149	0.174
			MDD	-1.004	0.317	0.007	-1.770	-0.239
	CXCL10	Control	MDD	-1.094	0.372	0.013	-1.991	-0.197
			BPD	-0.150	0.388	0.921	-1.084	0.784
		MDD	Control	1.094	0.372	0.013	0.197	1.991
			BPD	0.944	0.421	0.072	-0.068	1.956
		BPD	Control	0.150	0.388	0.921	-0.784	1.084
			MDD	-0.944	0.421	0.072	-1.956	0.068
	CXCL11	Control	MDD	-0.008	0.444	1.000	-1.102	1.086
			BPD	0.328	0.434	0.733	-0.731	1.386
		MDD	Control	0.008	0.444	1.000	-1.086	1.102
			BPD	0.336	0.430	0.718	-0.721	1.393
		BPD	Control	-0.328	0.434	0.733	-1.386	0.731
			MDD	-0.336	0.430	0.718	-1.393	0.721
	CXCL12	Control	MDD	0.012	0.342	0.999	-0.857	0.880
			BPD	-0.197	0.430	0.892	-1.270	0.877
		MDD	Control	-0.012	0.342	0.999	-0.880	0.857
			BPD	-0.208	0.374	0.844	-1.153	0.736
		BPD	Control	0.197	0.430	0.892	-0.877	1.270
			MDD	0.208	0.374	0.844	-0.736	1.153
	CXCL13	Control	MDD	-0.173	0.316	0.849	-0.937	0.592
			BPD	-0.106	0.316	0.940	-0.874	0.661
		MDD	Control	0.173	0.316	0.849	-0.592	0.937
			BPD	0.066	0.329	0.978	-0.733	0.865
		BPD	Control	0.106	0.316	0.940	-0.661	0.874
			MDD	-0.066	0.329	0.978	-0.865	0.733
Chemokine receptor	CCR1	Control	MDD	-0.392	0.227	0.202	-0.938	0.153
			BPD	-0.385	0.216	0.184	-0.903	0.134
		MDD	Control	0.392	0.227	0.202	-0.153	0.938
			BPD	0.008	0.233	0.999	-0.552	0.567
		BPD	Control	0.385	0.216	0.184	-0.134	0.903
			MDD	-0.008	0.233	0.999	-0.567	0.552

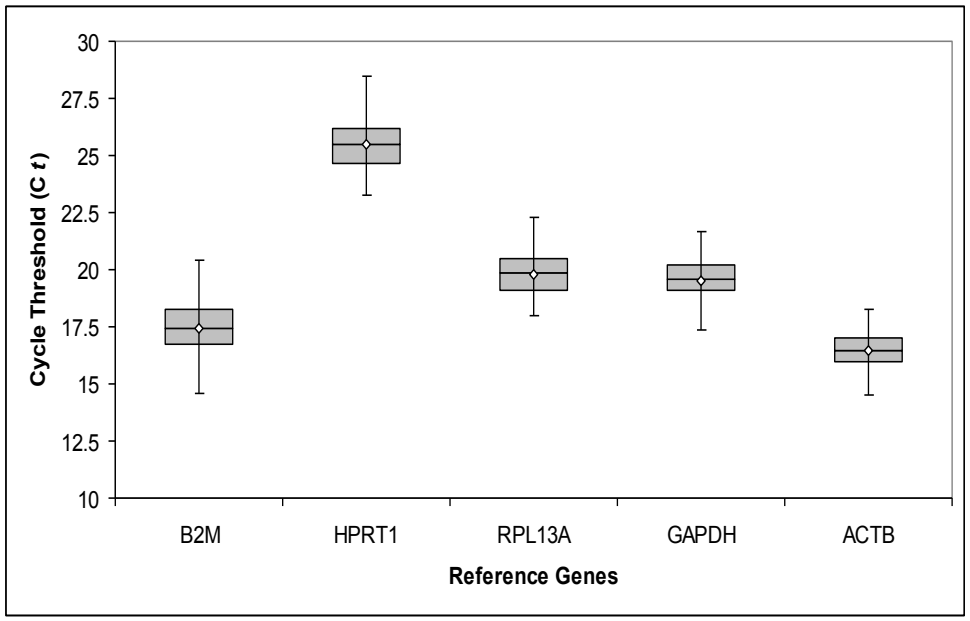
	CCR2	Control	MDD	-0.204	0.261	0.717	-0.832	0.424
			BPD	-0.460	0.337	0.366	-1.274	0.353
		MDD	Control	0.204	0.261	0.717	-0.424	0.832
			BPD	-0.257	0.332	0.721	-1.058	0.544
		BPD	Control	0.460	0.337	0.366	-0.353	1.274
			MDD	0.257	0.332	0.721	-0.544	1.058
	CCR3	Control	MDD	-0.363	0.371	0.593	-1.257	0.530
			BPD	-0.181	0.373	0.878	-1.079	0.716
		MDD	Control	0.363	0.371	0.593	-0.530	1.257
			BPD	0.182	0.343	0.857	-0.644	1.008
		BPD	Control	0.181	0.373	0.878	-0.716	1.079
			MDD	-0.182	0.343	0.857	-1.008	0.644
	CCR4	Control	MDD	-0.615	0.255	0.049	-1.229	-0.001
			BPD	-0.108	0.236	0.891	-0.676	0.460
		MDD	Control	0.615	0.255	0.049	0.001	1.229
			BPD	0.507	0.278	0.170	-0.161	1.175
		BPD	Control	0.108	0.236	0.891	-0.460	0.676
			MDD	-0.507	0.278	0.170	-1.175	0.161
	CCR5	Control	MDD	-0.057	0.231	0.967	-0.614	0.500
			BPD	-0.174	0.291	0.822	-0.875	0.527
		MDD	Control	0.057	0.231	0.967	-0.500	0.614
			BPD	-0.117	0.269	0.900	-0.766	0.532
		BPD	Control	0.174	0.291	0.822	-0.527	0.875
			MDD	0.117	0.269	0.900	-0.532	0.766
	CCR6	Control	MDD	-0.510	0.192	0.028	-0.973	-0.047
			BPD	-0.419	0.205	0.111	-0.913	0.074
		MDD	Control	0.510	0.192	0.028	0.047	0.973
			BPD	0.091	0.215	0.907	-0.426	0.607
		BPD	Control	0.419	0.205	0.111	-0.074	0.913
			MDD	-0.091	0.215	0.907	-0.607	0.426
	CCR7	Control	MDD	-0.553	0.242	0.066	-1.136	0.030
			BPD	-0.345	0.213	0.247	-0.859	0.169
		MDD	Control	0.553	0.242	0.066	-0.030	1.136
			BPD	0.208	0.256	0.697	-0.409	0.825
		BPD	Control	0.345	0.213	0.247	-0.169	0.859
			MDD	-0.208	0.256	0.697	-0.825	0.409
	CCR8	Control	MDD	-0.402	0.486	0.689	-1.584	0.781
			BPD	0.001	0.294	1.000	-0.707	0.708
		MDD	Control	0.402	0.486	0.689	-0.781	1.584
			BPD	0.402	0.496	0.698	-0.802	1.606
		BPD	Control	-0.001	0.294	1.000	-0.708	0.707
			MDD	-0.402	0.496	0.698	-1.606	0.802
	CCR9	Control	MDD	-0.644	0.249	0.032	-1.242	-0.046
			BPD	-0.368	0.256	0.327	-0.983	0.247
		MDD	Control	0.644	0.249	0.032	0.046	1.242
			BPD	0.276	0.247	0.507	-0.318	0.870
		BPD	Control	0.368	0.256	0.327	-0.247	0.983
			MDD	-0.276	0.247	0.507	-0.870	0.318
	CX3CR1	Control	MDD	-0.280	0.314	0.648	-1.035	0.475
			BPD	-0.012	0.335	0.999	-0.817	0.793
		MDD	Control	0.280	0.314	0.648	-0.475	1.035
			BPD	0.268	0.310	0.666	-0.479	1.014
		BPD	Control	0.012	0.335	0.999	-0.793	0.817
			MDD	-0.268	0.310	0.666	-1.014	0.479

Gene Family	Gene	Subject Group	Comparison Group	Mean Difference	S.E.	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
	XCR1	Control	MDD	0.109	0.226	0.881	-0.438	0.656
			BPD	-0.664	0.214	0.009	-1.180	-0.148
		MDD	Control	-0.109	0.226	0.881	-0.656	0.438
			BPD	-0.772	0.262	0.013	-1.404	-0.141
		BPD	Control	0.664	0.214	0.009	0.148	1.180
			MDD	0.772	0.262	0.013	0.141	1.404
Interleukin	IL1A	Control	MDD	-0.410	0.455	0.642	-1.511	0.690
			BPD	-0.444	0.487	0.635	-1.625	0.737
		MDD	Control	0.410	0.455	0.642	-0.690	1.511
			BPD	-0.034	0.447	0.997	-1.121	1.053
		BPD	Control	0.444	0.487	0.635	-0.737	1.625
			MDD	0.034	0.447	0.997	-1.053	1.121
	IL1B	Control	MDD	-0.469	0.246	0.147	-1.061	0.124
			BPD	-0.346	0.273	0.420	-1.003	0.311
		MDD	Control	0.469	0.246	0.147	-0.124	1.061
			BPD	0.123	0.254	0.879	-0.488	0.733
		BPD	Control	0.346	0.273	0.420	-0.311	1.003
			MDD	-0.123	0.254	0.879	-0.733	0.488
	IL5	Control	MDD	-0.405	0.400	0.573	-1.369	0.559
			BPD	-0.204	0.390	0.861	-1.145	0.737
		MDD	Control	0.405	0.400	0.573	-0.559	1.369
			BPD	0.201	0.328	0.814	-0.588	0.990
		BPD	Control	0.204	0.390	0.861	-0.737	1.145
			MDD	-0.201	0.328	0.814	-0.990	0.588
	IL6	Control	MDD	-0.589	0.328	0.180	-1.379	0.200
			BPD	-0.107	0.300	0.933	-0.829	0.616
		MDD	Control	0.589	0.328	0.180	-0.200	1.379
			BPD	0.483	0.315	0.284	-0.277	1.243
		BPD	Control	0.107	0.300	0.933	-0.616	0.829
			MDD	-0.483	0.315	0.284	-1.243	0.277
	IL8	Control	MDD	-1.021	0.303	0.004	-1.749	-0.292
			BPD	-0.995	0.316	0.007	-1.756	-0.234
		MDD	Control	1.021	0.303	0.004	0.292	1.749
			BPD	0.026	0.323	0.997	-0.750	0.802
		BPD	Control	0.995	0.316	0.007	0.234	1.756
			MDD	-0.026	0.323	0.997	-0.802	0.750
	IL10	Control	MDD	-0.432	0.314	0.362	-1.190	0.327
			BPD	0.037	0.247	0.987	-0.556	0.631
		MDD	Control	0.432	0.314	0.362	-0.327	1.190
			BPD	0.469	0.311	0.295	-0.281	1.220
		BPD	Control	-0.037	0.247	0.987	-0.631	0.556
			MDD	-0.469	0.311	0.295	-1.220	0.281
	IL11	Control	MDD	-0.029	0.433	0.998	-1.088	1.031
			BPD	-0.575	0.525	0.526	-1.887	0.737
		MDD	Control	0.029	0.433	0.998	-1.031	1.088
			BPD	-0.546	0.532	0.567	-1.868	0.775
		BPD	Control	0.575	0.525	0.526	-0.737	1.887
			MDD	0.546	0.532	0.567	-0.775	1.868
	IL13	Control	MDD	-0.071	0.369	0.980	-0.972	0.830
			BPD	0.964	0.789	0.454	-1.028	2.955
		MDD	Control	0.071	0.369	0.980	-0.830	0.972
			BPD	1.034	0.801	0.415	-0.980	3.049
		BPD	Control	-0.964	0.789	0.454	-2.955	1.028
			MDD	-1.034	0.801	0.415	-3.049	0.980

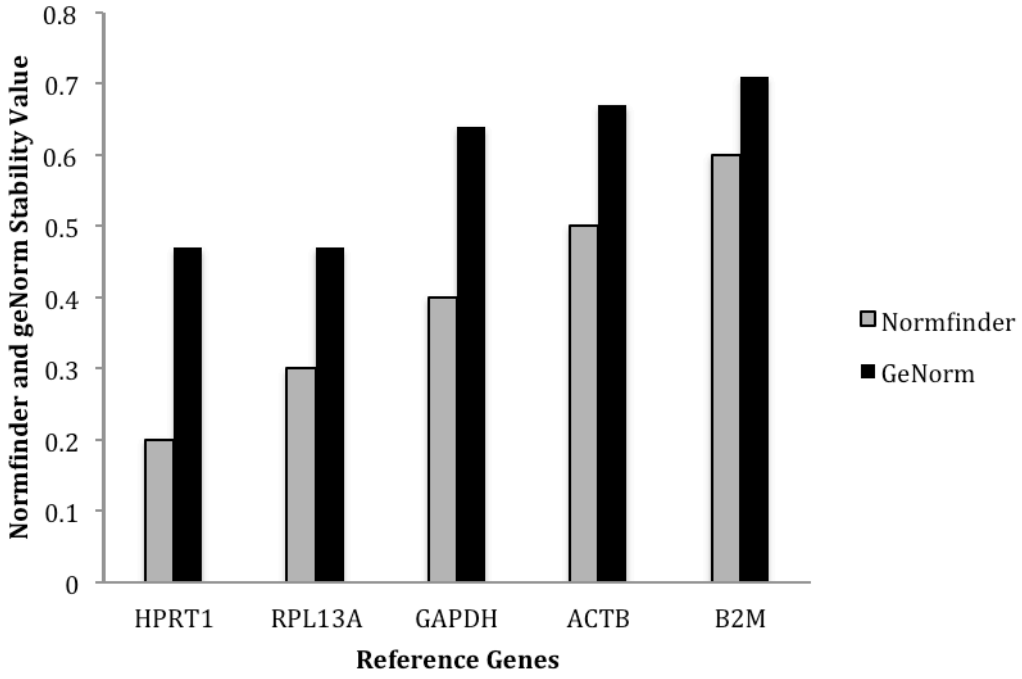
Gene Family	Gene	Subject Group	Comparison Group	Mean Difference	S.E.	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
	IL17C	Control	MDD	-0.018	0.235	0.997	-0.583	0.548
			BPD	-0.172	0.273	0.805	-0.828	0.485
		MDD	Control	0.018	0.235	0.997	-0.548	0.583
			BPD	-0.154	0.257	0.820	-0.773	0.464
		BPD	Control	0.172	0.273	0.805	-0.485	0.828
			MDD	0.154	0.257	0.820	-0.464	0.773
	IL22	Control	MDD	0.168	0.331	0.868	-0.634	0.970
			BPD	-0.319	0.345	0.628	-1.155	0.517
		MDD	Control	-0.168	0.331	0.868	-0.970	0.634
			BPD	-0.487	0.333	0.320	-1.295	0.322
		BPD	Control	0.319	0.345	0.628	-0.517	1.155
			MDD	0.487	0.333	0.320	-0.322	1.295
	IL36A	Control	MDD	0.009	0.325	1.000	-0.778	0.796
			BPD	0.017	0.251	0.997	-0.589	0.623
		MDD	Control	-0.009	0.325	1.000	-0.796	0.778
			BPD	0.008	0.305	1.000	-0.734	0.750
		BPD	Control	-0.017	0.251	0.997	-0.623	0.589
			MDD	-0.008	0.305	1.000	-0.750	0.734
	IL37	Control	MDD	0.304	0.339	0.646	-0.517	1.125
			BPD	-0.253	0.356	0.758	-1.113	0.607
		MDD	Control	-0.304	0.339	0.646	-1.125	0.517
			BPD	-0.557	0.410	0.369	-1.544	0.430
		BPD	Control	0.253	0.356	0.758	-0.607	1.113
			MDD	0.557	0.410	0.369	-0.430	1.544
Interleukin receptor	IL1R1	Control	MDD	-0.074	0.314	0.970	-0.828	0.681
			BPD	0.043	0.266	0.986	-0.597	0.683
		MDD	Control	0.074	0.314	0.970	-0.681	0.828
			BPD	0.117	0.294	0.917	-0.592	0.826
		BPD	Control	-0.043	0.266	0.986	-0.683	0.597
			MDD	-0.117	0.294	0.917	-0.826	0.592
	IL1RN	Control	MDD	-0.206	0.230	0.645	-0.759	0.347
			BPD	-0.246	0.221	0.511	-0.778	0.287
		MDD	Control	0.206	0.230	0.645	-0.347	0.759
			BPD	-0.040	0.236	0.984	-0.608	0.528
		BPD	Control	0.246	0.221	0.511	-0.287	0.778
			MDD	0.040	0.236	0.984	-0.528	0.608
	IL5RA	Control	MDD	-0.249	0.285	0.659	-0.935	0.437
			BPD	0.273	0.255	0.536	-0.341	0.887
		MDD	Control	0.249	0.285	0.659	-0.437	0.935
			BPD	0.522	0.271	0.140	-0.130	1.175
		BPD	Control	-0.273	0.255	0.536	-0.887	0.341
			MDD	-0.522	0.271	0.140	-1.175	0.130
	IL8RA	Control	MDD	-0.123	0.224	0.846	-0.662	0.415
			BPD	-0.333	0.244	0.364	-0.920	0.253
		MDD	Control	0.123	0.224	0.846	-0.415	0.662
			BPD	-0.210	0.234	0.644	-0.773	0.353
		BPD	Control	0.333	0.244	0.364	-0.253	0.920
			MDD	0.210	0.234	0.644	-0.353	0.773
	IL8RB	Control	MDD	-0.021	0.240	0.996	-0.598	0.556
			BPD	-0.352	0.272	0.404	-1.007	0.302
		MDD	Control	0.021	0.240	0.996	-0.556	0.598
			BPD	-0.331	0.266	0.431	-0.971	0.308
		BPD	Control	0.352	0.272	0.404	-0.302	1.007
			MDD	0.331	0.266	0.431	-0.308	0.971

Gene Family	Gene	Subject Group	Comparison Group	Mean Difference	S.E.	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
	IL9R	Control	MDD	0.173	0.487	0.933	-1.061	1.407
			BPD	-0.437	0.319	0.371	-1.231	0.356
		MDD	Control	-0.173	0.487	0.933	-1.407	1.061
			BPD	-0.611	0.502	0.456	-1.873	0.652
		BPD	Control	0.437	0.319	0.371	-0.356	1.231
			MDD	0.611	0.502	0.456	-0.652	1.873
	IL10RA	Control	MDD	-0.205	0.165	0.433	-0.603	0.192
			BPD	-0.243	0.183	0.388	-0.685	0.199
		MDD	Control	0.205	0.165	0.433	-0.192	0.603
			BPD	-0.038	0.188	0.978	-0.490	0.415
		BPD	Control	0.243	0.183	0.388	-0.199	0.685
			MDD	0.038	0.188	0.978	-0.415	0.490
	IL10RB	Control	MDD	-0.187	0.218	0.668	-0.713	0.338
			BPD	-0.136	0.236	0.835	-0.706	0.435
		MDD	Control	0.187	0.218	0.668	-0.338	0.713
			BPD	0.052	0.259	0.978	-0.571	0.674
		BPD	Control	0.136	0.236	0.835	-0.435	0.706
			MDD	-0.052	0.259	0.978	-0.674	0.571
	IL13RA1	Control	MDD	-0.211	0.210	0.575	-0.716	0.293
			BPD	-0.193	0.221	0.657	-0.724	0.337
		MDD	Control	0.211	0.210	0.575	-0.293	0.716
			BPD	0.018	0.228	0.997	-0.530	0.566
		BPD	Control	0.193	0.221	0.657	-0.337	0.724
			MDD	-0.018	0.228	0.997	-0.566	0.530
Tumor necrosis factor	TNF	Control	MDD	-0.023	0.154	0.987	-0.397	0.351
			BPD	-0.198	0.168	0.474	-0.604	0.208
		MDD	Control	0.023	0.154	0.987	-0.351	0.397
			BPD	-0.174	0.124	0.347	-0.474	0.125
		BPD	Control	0.198	0.168	0.474	-0.208	0.604
			MDD	0.174	0.124	0.347	-0.125	0.474
	LTA	Control	MDD	-0.183	0.191	0.608	-0.644	0.279
			BPD	-0.292	0.230	0.416	-0.845	0.260
		MDD	Control	0.183	0.191	0.608	-0.279	0.644
			BPD	-0.110	0.192	0.836	-0.575	0.355
		BPD	Control	0.292	0.230	0.416	-0.260	0.845
			MDD	0.110	0.192	0.836	-0.355	0.575
	LTB	Control	MDD	-0.130	0.190	0.775	-0.589	0.330
			BPD	-0.272	0.239	0.496	-0.847	0.304
		MDD	Control	0.130	0.190	0.775	-0.330	0.589
			BPD	-0.142	0.203	0.764	-0.633	0.349
		BPD	Control	0.272	0.239	0.496	-0.304	0.847
	CD40LG	Control	MDD	0.010	0.150	0.998	-0.352	0.372
			BPD	-0.122	0.170	0.755	-0.530	0.287
		MDD	Control	-0.010	0.150	0.998	-0.372	0.352
			BPD	-0.131	0.156	0.677	-0.507	0.244
		BPD	Control	0.122	0.170	0.755	-0.287	0.530
			MDD	0.131	0.156	0.677	-0.244	0.507
	LTB4R	Control	MDD	-0.359	0.235	0.289	-0.930	0.212
			BPD	-0.504	0.279	0.177	-1.176	0.168
		MDD	Control	0.359	0.235	0.289	-0.212	0.930
			BPD	-0.145	0.215	0.779	-0.666	0.376
		BPD	Control	0.504	0.279	0.177	-0.168	1.176
			MDD	0.145	0.215	0.779	-0.376	0.666

Gene Family	Gene	Subject Group	Comparison Group	Mean Difference	S.E.	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
Other	AIMP1	Control	MDD	-0.298	0.192	0.277	-0.761	0.165
			BPD	-0.291	0.276	0.546	-0.959	0.376
		MDD	Control	0.298	0.192	0.277	-0.165	0.761
			BPD	0.006	0.266	1.000	-0.640	0.652
		BPD	Control	0.291	0.276	0.546	-0.376	0.959
			MDD	-0.006	0.266	1.000	-0.652	0.640
	BCL6	Control	MDD	-0.158	0.274	0.833	-0.818	0.501
			BPD	-0.370	0.258	0.330	-0.993	0.252
		MDD	Control	0.158	0.274	0.833	-0.501	0.818
			BPD	-0.212	0.224	0.614	-0.753	0.328
		BPD	Control	0.370	0.258	0.330	-0.252	0.993
			MDD	0.212	0.224	0.614	-0.328	0.753
	CEBPB	Control	MDD	-0.213	0.283	0.734	-0.894	0.469
			BPD	0.161	0.291	0.845	-0.540	0.862
		MDD	Control	0.213	0.283	0.734	-0.469	0.894
			BPD	0.374	0.281	0.383	-0.301	1.049
		BPD	Control	-0.161	0.291	0.845	-0.862	0.540
			MDD	-0.374	0.281	0.383	-1.049	0.301
	MIF	Control	MDD	-0.233	0.191	0.451	-0.697	0.232
			BPD	-0.346	0.245	0.341	-0.934	0.243
		MDD	Control	0.233	0.191	0.451	-0.232	0.697
			BPD	-0.113	0.194	0.831	-0.585	0.359
		BPD	Control	0.346	0.245	0.341	-0.243	0.934
			MDD	0.113	0.194	0.831	-0.359	0.585
	NR3C1	Control	MDD	-0.543	0.202	0.025	-1.028	-0.057
			BPD	-0.373	0.222	0.221	-0.906	0.161
		MDD	Control	0.543	0.202	0.025	0.057	1.028
			BPD	0.170	0.208	0.696	-0.332	0.672
		BPD	Control	0.373	0.222	0.221	-0.161	0.906
			MDD	-0.170	0.208	0.696	-0.672	0.332
	SPP1	Control	MDD	0.537	0.476	0.502	-0.610	1.684
			BPD	-0.405	0.500	0.697	-1.607	0.797
		MDD	Control	-0.537	0.476	0.502	-1.684	0.610
			BPD	-0.943	0.456	0.105	-2.039	0.154
		BPD	Control	0.405	0.500	0.697	-0.797	1.607
			MDD	0.943	0.456	0.105	-0.154	2.039
	TOLLIP	Control	MDD	0.123	0.149	0.689	-0.235	0.481
			BPD	-0.016	0.133	0.992	-0.335	0.303
		MDD	Control	-0.123	0.149	0.689	-0.481	0.235
			BPD	-0.139	0.156	0.649	-0.515	0.237
		BPD	Control	0.016	0.133	0.992	-0.303	0.335
			MDD	0.139	0.156	0.649	-0.237	0.515



S1: Real-time PCR cycle threshold values (Ct) in five reference genes in blood from depressed patients both at baseline and after eight weeks of treatment with escitalopram. Expression levels are shown as median (lines), 25th percentile to the 75th percentile (boxes), ranges (whiskers) and means (white diamonds).



S2: A graph showing the results of the NormFinder and geNorm analyses. The x-axis shows the reference genes tested and the y-axis shows their Normfinder and geNorm* stability values. The lower the stability values the more stable the reference gene. *HPRT1* was shown to be the most stable reference genes following escitalopram treatment, and *B2M* the most unstable reference gene. Note: geNorm stability values have been scaled up by a factor of 10^2.

Week 0	Gene	r	p-value
	ABCF1	0.492	0.001*
	C5	0.451	0.002*
	CCL4	0.575	278E-05*
	CXCL11	0.331	0.08
	IL10RA	0.727	1.55E-08*
	LTA	0.341	0.02*
	CCR9	-1.52	0.313
Week 8	IL11	0.225	0.216
	CCL24	0.413	0.004*
	CCL26	0.269	0.113
	CCL4	0.528	1.64E-04*
	LTA	0.488	0.001*
	MIF	0.482	0.001*
	B2M	0.679	2.08E-07*

S3: Table detailing the top gene expression differences between responders and non-responders at week 0 and week 8 and their correlation with the expression of *TNF* at each week. R-values represent the correlation obtained from a two-direction Pearson's correlation test, and the p-values the significance of the correlation.